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A New Apparatus for Long Term Measurement of Oxygen Consumption in Small Mammals. (21858)

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Numerous respirometers have been designed for measurement of oxygen consumption, but few are suitable for measurements of long duration(1,2,3); none is capable of automatically correcting for changes in barometric pressure during continuous recording(4). In experiments with hibernating bats, in which long periods of observation were necessary and in which the oxygen consumption was very small, it was difficult to make corrections for pressure. For instance, a 2% change in barometric pressure represents a 20 ml change in a liter animal chamber, an amount which may be greater than the oxygen consumed by the animal during the same period.

The present paper reports the development of a new closed-circuit apparatus for continuous recording of oxygen consumption over periods of several days with automatic correction for changes in barometric pressure. The instrument is sensitive to less than 0.01 ml of oxygen consumption per hour, and will measure oxygen consumption over a wide range.

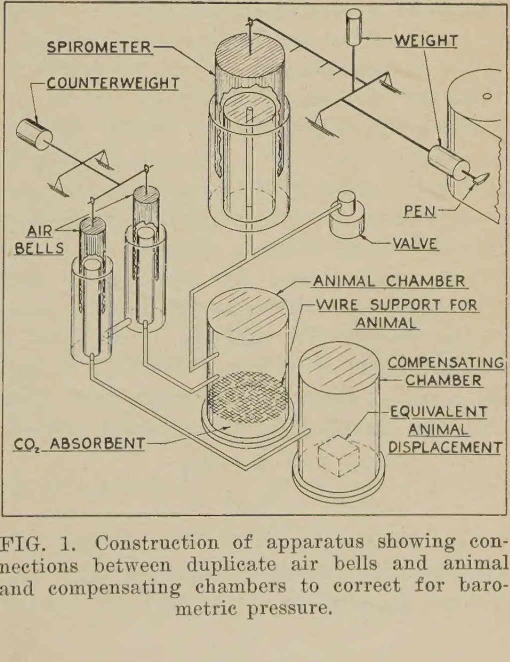

FIG. 1. Construction of apparatus showing connections between duplicate air bells and animal and compensating chambers to correct for barometric pressure.

Description of Apparatus. A diagram of the apparatus is shown in Fig. 1. The animal is placed in a plastic chamber of 660 ml capacity. The top, containing the animal within a wire framework, fits into the base in a groove which is brushed with triacetin to form a gas-tight seal. The hollow base 85 mm in diameter contains a solution of sodium hydroxide for the absorption of carbon dioxide. The animal chamber is connected to a spirometer containing 50 ml of oxygen. The animal chamber is also connected to one of 2 similar air bells which are immersed in a water bath, and which operate together on one end of a counter-balanced lever. The second air bell is connected to a pressure compensating chamber of the same size, shape and material as the animal chamber described above.

Changes in barometric pressure during a period of recording are compensated for by expansion or contraction of air in the compensating chamber. This results in the adding or subtracting of a corresponding volume in the animal chamber by movement of the air bells. Hence, there is little movement of the spirometer as a result of changes in atmospheric pressure. For example, when the barometric pressure drops from 740 mm to 720 mm, the volume of the air in the compensating chamber increases almost 20 ml. This increase in volume is made possible by the rise of the air bell connected to the compensating chamber. Since both air bells are attached to the same lever, the one attached to the animal chamber rises at the same time. Therefore, the compensating device greatly reduces the rise in the spirometer which would be recorded as an apparent decrease in oxygen consumption, when the barometric pressure drops. Compensation is not made for the volume of gas in the spirometer; the error depends not on the actual volume of oxygen used but on the oxygen levels at the beginning and end of a period of measurement. The error is greatest when the spirometer is filled with oxygen and decreases to zero as the spirometer empties. In practice, the error is calculated by multiplying the mean level of oxygen in the spirometer times the percentage change in barometric pressure during a given interval. The oxygen levels at any time can be read from the record and the pressures from a barometric record. For example, if the level in the spirometer drops from 50 ml to 0 ml during a period when the barometer drops from 740 mm to 720 mm the error is 0.67 ml oxygen $\left(\frac{50\text{-}0}{2} \text{ x } \frac{740\text{-}720}{740} \right)$ and the actual oxygen consumption is 50.7 ml. Without the compensating chamber, under the same conditions, the error would be 18.5 ml. If calculations of the error are not made, a bottle of 25 ml capacity (half the volume of the spirometer) may be put in the line between the compensating chamber and air bell as an approximation for the volume of the gas in the spirometer.

The air bells and spirometer are made from brass tubing turned down to a thickness of 0.005 inch and then plated with copper oxide to reduce surface tension. The spirometer has a diameter of 2¼ inches and a length of 4 inches and displaces less than one milliliter; the air bells displace even less. These very small displacements facilitate balancing the spirometer and air bells with counterweights so that they stay in any position in which they are set. The water surface within each bell is kept to a minimum by fitting each bell over an inner plastic surface ¼ inch smaller in diameter than the bell itself.

The apparatus is sensitive to small changes in gas volume, recording pressure changes of 1 millibar. This sensitivity is achieved by making the water surface very small, and by decreasing its surface tension with a wetting agent and by coating the bells with copper oxide. Furthermore, condensation on the inside of the bells is prevented by 5% ethylene glycol in the water which keeps the vapor pressure below the dewpoint and prevents bacterial growth.

The present instrument was designed so that when the 50 ml spirometer is used, a vertical distance of 1 inch on the record represents 16 ml of oxygen consumption. The paper moves at a rate of one-half inch per hour, although other gears may be used to vary the speed. For convenience, standard curves have been drawn on thin plastic sheets. These may be placed over the record and the rate of oxygen consumption read directly in ml/hour for any part of the curve.

The design of the present apparatus offers several additional advantages. The apparatus is readily portable and experiments are run in different constant temperature rooms. Leakage of gas has been practically eliminated since the gas train is composed entirely of plastic and copper. However, leakage is routinely checked by running a blank for several hours before and after each experiment

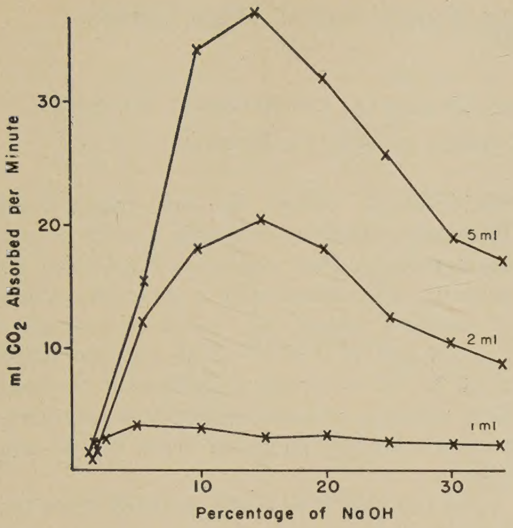

FIG. 2. Carbon dioxide absorption by 1, 2 and 5 ml of solutions of different concentrations of NaOH from 1% to 36% placed in the base of the 660 ml animal chamber at 27°C.

during which period the line on the record should remain horizontal. In addition, different sized chambers and spirometers are readily interchangeable for use with different species.

Carbon dioxide is absorbed by a solution of sodium hydroxide placed in the base of the animal chamber. In order to determine the capacity for carbon dioxide absorption in this animal chamber, the absorption of known concentrations of carbon dioxide was measured. Before each determination the air in the spirometer was removed and replaced with carbon dioxide. Measured quantities of several different concentrations of sodium hydroxide were placed in the animal chamber and the apparatus was allowed to reach equilibrium over a period of 5 minutes. Then 5 ml of air was withdrawn from the chamber by a syringe and was automatically replaced by carbon dioxide from the spirometer. As carbon dioxide was subsequently absorbed from the animal chamber its concentration remained constant because an equal volume of carbon dioxide was added from the spirometer. Fig. 2 shows the results obtained with various amounts of different concentrations from 1 to 36% sodium hydroxide at 27°C. Similar curves were obtained at 5°C and 37°C and with the carbon dioxide level at 10 ml at the same temperatures. Hitchcock(5) found that the rate of absorption is proportional to the carbon dioxide gradient and inversely proportional to the liquid viscosity, since the predominant factor in determining the rate of absorption was the diffusion of carbon dioxide through the liquid. In this apparatus the entire base of the animal chamber provides a large area for absorption. Since, for example, 5 ml of fresh 15% sodium hydroxide absorbs about 40 ml of carbon dioxide per minute from the 660 ml animal chamber, the absorptive capacity greatly exceeds the rate of carbon dioxide production of the mammals studied. In addition, the base may be replaced with another containing fresh sodium hydroxide without disturbing the animal.

Summary. A new closed-circuit apparatus has been described which continuously records oxygen consumption over periods of several days with automatic correction for changes in barometric pressure. The equipment is sensitive to less than 0.01 ml of oxygen per hour and can measure oxygen consumption over a wide range.

I am very grateful to Mr. C. F. Wallace for his assistance in designing and building the apparatus described.

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Virolysin: A Virus-Induced Lysin from Staphylococcal Phage Lysates.*

(21859)

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Non-phage lytic agents present in phage lysates have been described by various investigators(1-6).

A lysin, for which the term "virolysin" is proposed, has been detected in phage P14-*S. aureus* K_1 lysates. This lysin appears during phage infection and is similar to but not identical with the autolysin of uninfected host cells. The evidence suggests that in conjunction with virus material it can cause an abnormal or premature lysis of viable host cells and that it also functions in the final lysis that normally follows infection of host cells.

Procedures. Host-Phage Systems: Phages P_1 and P_{14} and their hosts, *S. aureus* K_1 and 145, and their method of assay have been described(7,8). Most of the studies reported herein were carried out with the phage P14-host K_1 system, 14 (K_1). *Phage Lysates*: Crude lysates were prepared by inoculating double strength tryptose-phosphate broth (2 x TP), Difco, with a 24-hour agar-grown K_1 cell suspension to a concentration of 1 x 10^8 cells/ml and phage 14 at 1 x 10^6 particles/ml. Flasks were shaken at 37°C until lysis (usually 3 hr.) *Cell Autolysates*: An initial inoculum of 1 x 10^8/ml resting cells of *S. aureus* K_1 was shaken at 37°C in 2 x TP broth for 8 hours. Flasks were stored at 4°C for a minimum of 6-7 days in order to obtain release of active autolysin. *Ultracentrifugation*: Both phage lysates and cell autolysates were centrifuged at 35,000 g for one hour at 6°C in a Spinco, model L ultracentrifuge. The supernatant layers were decanted and tested for lytic activity. The pellets were resuspended in M/15 phosphate buffer. *Measurement of Lysis*: Estimation of lysin activity was based on the rate of lysis of standard heat-killed *S. aureus* K_1 cell preparations. Routinely, dilutions of phage lysates or cell autolysates, or their respective high-speed supernates, were mixed with a constant number of standard heated cells (final concentration 6 x 10^8/ml) in 2 x TP broth and incubated at 37°C. At intervals, turbidimetric readings were made in a Klett Summerson photoelectric colorimeter, equipped with a 660 mu filter.

The per cent lysis was calculated from the drop in Klett units in a given time interval. *Cell preparations.* a. *Heat-killed cells.* Standard cells were prepared by harvesting logarithmic phase TP agar-grown cells in 0.85% saline to ca 5 x 10^{10}/ml and heating at 56°C for 1 hr. b. *Acetonized cells.* Cells were harvested from TP agar plates into saline and treated at room temperature with approximately 4 volumes of acetone for 10 to 20 minutes. The cells were centrifuged off at low speed, dried, and resuspended in physiological saline to approximately 5 x 10^{10}/ml.

Results. When sufficient amounts of crude phage P14(K_1) lysate are mixed with viable host K_1 cells at 3 x 10^8/ml in a broth medium, a lysis ensues which differs from that normally following phage production. (1) Lysis of viable cells begins immediately (within 15 minutes) at 37°C without the intervention of a typical latent period; (2) lysis proceeds in the cold at 0°C, a temperature which does not support phage production. It is accompanied by loss of initial phage, and (3) crude phage lysates are capable of lysing heat-killed cells, both at 37° and 4°C (Table I).

I. *Separation of Virolysin from Phage.* Ultracentrifugation is sufficient to separate the lytic system of crude phage 14(K_1) lysate into 2 components: a pellet, containing active virus and a supernate, containing the lysin, virolysin. Active phage (or pellet) alone fails to cause premature lysis of viable cells at 37°C and has no action on acetonized or heat-killed cells. (Table II). Recombination

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TABLE I. Lytic Activity of Crude Phage Broth Lysates for *S. aureus* K_1 Preparations.

A. Viable cells

Lysis at 37°C		Lysis at 4°C				
Dilution of lysate,[*] 14(K_1)	% lysis[†] in 15 min. (input 5 × 10^8 cells/ml)	Dilution of lysate, 14(K_1)	% lysis in 24 hr (input 4 × 10^8 cells/ml)	Phage input, plaques/ml	Phage residual after 24 hr, plaques/ml	Phage adsorbed/cell, P/B
Undil.	57	1: 1.11	83	1.1 × 10^{10}	3.0 × 10^9	22
1: 2	53	1: 1.8	83	6.7 × 10^9	6.8 × 10^8	15
1: 5	16	1: 3.6	78	3.4 × 10^9	9.0 × 10^6	9
1:10	0[‡]	1: 5.9	65	2.0 × 10^9	3.0 × 10^7	5
1:20	0[‡]	1:16.7	45	7.2 × 10^8	4.7 × 10^7	2

B. Heat killed cells

Lysis at 37°C		Lysis at 4°C	
Dilution of lysate, 14(K_1)	% lysis in 20 min. (input 6 × 10^8 cells/ml)	Dilution of lysate, 14(K_1)	% lysis in 24 hr (input 6 × 10^8 cells/ml)
Undil.	63	Undil.	36
1: 5	21	1: 2	26
1:10	11	1: 5	22
1:20	9.5	1:10	14
1:40	8.8	1:20	6

Five ml lysate, diluted in TP2x broth was mixed with 0.5 ml cell preparation in a Klett-size pyrex tube. Viable cells were harvested during the exponential phase of growth on TP agar.

[*] Lysate preparations reported were from different lots, so that cross comparison of activity is not possible.

[†] The latent period of normal phage 14(K_1) infection of such cells was found to be 35-38 min. at 37°C, as estimated by infection of 5 × 10^8 ml cells with an ultracentrifuged phage 14(K_1) preparation at a P/B ratio = 3/1, in TP2x broth at 37°C and Klett turbidimetric determination of the onset of lysis.

[‡] Inhibition of cell growth occurred.

of the pellet and the supernate (virolysin) restores ability to cause premature lysis of viable cells. As few as one or 2 particles adsorbed per viable cell, together with the enzyme, cause extensive lysis, even in the cold. (Table I).

II. *Comparison of Virolysin and Autolysin.* The properties of material present in the high-speed supernates of crude phage 14 (K_1) lysate and of uninfected *S. aureus* K_1 cell autolysate were found to differ with respect to their pH optima for lysis of heat-killed K_1 cells and also their relative lytic specificity for *M. lysodeikticus.* The optimum for lysis of heat-killed K_1 cells at 37°C by autolysin is between pH 6.0 and 6.5, with a rapid loss of activity above pH 7.5 to 8.0, whereas the optimum for virolysin lies between pH 7.0 and 7.5, with a rapid decrease of activity below pH 6.5. Furthermore, when preparations containing active virolysin and autolysin are mixed and tested over a range from pH 6.5 to 8.5, the combined lysis is equal to the sum of the individual enzyme ac-

TABLE II. Effect of Phage Lysate and Its Ultracentrifuged Components on Various Cell Preparations of *S. aureus* K_1.

	% lysis 35 min. 37°C (cell input 6 × 10^8/ml)					
	Stationary phase			Logarithmic phase		
Component tested	Viable	Heated	Acetonized	Viable	Heated	Acetonized
Crude lysate 14(K_1)	21	27	28	50	54	56
Supernate (virolysin)	5	26	26	0	53	55
Pellet (phage)	4	0	0	9	0	0
Recombined supernate and pellet	22	25	29	49	55	55

Conditions of experiment: 37°C, pH 7.2, dilutions in TP2x.

TABLE III. Lysis of Heated *S. aureus* K_1 by 14(K_1) Virolysin, K_1 Autolysin and Their Mixture at pH's 6.5, 7.5, 8.5.

		% cells lysed*		
		pH†		
Samples	Lysin	6.5	7.5	8.5
A	Autolysin	19	11	0
B	Virolysin	16	23	13
C	Autolysin + virolysin	39	31	20
D	Calculated for A + B	35	34	13

* At 37°C in 1 hr; initial cell concentration = 8.5×10^8 cells/ml.

† pH adjusted with 1 N NaOH or HCl.

A, supernate of K_1 autolysate after ultracentrifugation, diluted 1:2 in boiled high-speed supernate of 14(K_1) phage lysate. B, supernate of 14 (K_1) phage lysate after ultracentrifugation, diluted 1:2 in boiled high-speed supernate of K_1 autolysate. C, high-speed supernates of K_1 autolysate and of 14(K_1) phage lysate, mixed in equal parts.

tivities, indicating that neither preparation contains any substance influencing the lytic behaviour of the other. (Table III).

Table IV presents data to show that autolysin causes significant lysis of certain preparations of heat-killed *M. lysodeikticus*, whereas high concentrations of virolysin are relatively inert. The pH optimum for autolysin activity against *M. lysodeikticus* appears to be identical with its optimum for lysis of *S. aureus* K_1 cells, suggesting that the same enzyme in the autolysin preparation is acting on both hosts. Again, when the 2 enzymes are mixed and their lytic activities tested against killed cell preparations of *S. aureus* K_1 and *M. lysodeikticus* over a pH range from 6.0 to 8.0, as shown in Table IV, the lysis is additive, *i.e.*, equal to the sum of the individual activities.

The differences in pH optima and relative specificities for cell preparations of *M. lysodeikticus* appear to be basic enough to justify the conclusion that autolysin and virolysin are not identical. The exact nature of the difference awaits chemical purification of each enzyme. Evidence collected from their biological behaviour suggests that while the two enzymes behave differently as described above, nevertheless they exhibit certain common properties, suggesting that they are closely related. A summary of data follows: (1) Both materials are found in the supernate following centrifugation at 35,000 g for one hour. (2) Both lyse heat-killed homologous cells (56°C for 1 hr) and have no effect on fresh viable cells. Rate of lysis is roughly proportional to enzyme concentration. Concentration of virolysin in crude phage lysates appears to be considerably greater than that of autolysin present in cell autolysates. For example, they are active in dilutions up to 1/80 and 1/5 respectively. (3) Lysis proceeds in the cold. The Q_{10} for each material approximates 3.0. The value of u for both reactions is approximately 20,000 calories. (4) Both materials are thermolabile and under certain test conditions exhibit a rapid inactivation at 37°C over a pH range from 5.0 to 9.0, with a maximum heat-stability at pH 6.8. The crude autolysin, however, contains a thermolabile material (removed by boiling) which protects both enzymes against thermal inactivation at 37°C. (5) Purified trypsin, 2 µg/ml, destroys both materials. (6) Neither is inactivated by antiserum prepared with high-speed P_1 (related to P_{14}) concentrates. (7)

TABLE IV. Comparative Lysis of Heated *M. lysodeikticus* and Heated *S. aureus* K_1 by 14(K_1) Virolysin, K_1 Autolysin, and Their Mixture at pH's 6, 7, 8.

		% cells lysed								
		M. lysodeikticus			*S. aureus* K_1					
Sample	Lysin pH	6*	7*	8*	6†	7†	8†	6*	7*	8*
A	Autolysin	25	23	13	9	5	0	27	22	9
B	Virolysin	4	8	0	18	28	23	44	51	47
C	Autolysin + virolysin	38	32	15	30	39	25	44	53	50
D	Calculated from A + B	29	31	13	27	33	23	‡	‡	‡

* Readings taken at 5.5 hr.

† Readings taken at 2.1 hr.

‡ Not calculated; test has reached the end point where large majority of sensitive cells have been lysed.

Samples A, B, C, and conditions of experiment same as for Table III, except that lysis test was conducted at 23°C.

TABLE V. Observations on the Virus Nature of the Lytic Material in the Pellet Component of Ultracentrifuged Phage 14(K_1) Lysate. I. Correlation of phage titer and lytic activity. II. Ultraviolet inactivation of infectivity without loss of lytic activity.

I. Untreated pellet 14(K_1)

Test condition	Observation	Preparation No. 1	2	3
A. Tested at varying titer	Phage titer	1×10^{10}	5×10^9	1×10^9
	% lysis	66	50	38
B. Tested at constant titer	Phage titer	1.6×10^9	1.6×10^9	1.6×10^9
	% lysis	46	44	47
	Phage titer	2×10^8	2×10^8	2×10^8
	% lysis	26	27	27

II. UV irradiated pellet 14(K_1)

Observation	Min. UV irradiation 0	5	10
Phage titer	6×10^9	1×10^7	4×10^5
% lysis	46	44	46

I. Dilutions of pellet mixed with a constant volume of virolysin (high speed supernate of 14(K_1)) and logarithmic phase viable cells in TP2x broth at ca 6×10^8/ml. Percent lysis was calculated from Klett changes after 40 min. at 37°C.

II. Phage was irradiated in thin layer, with shaking, at a distance of 4 inches from a GE 15-watt Germicidal lamp. Lytic activity was tested as in I.

Both are adsorbed onto super-cel at pH 7.3. (8) They exhibit a similar host range against a taxonomically widely-distributed sample of heat-killed cells, lysing 25/25 strains of *S. aureus,* and to a lesser extent *S. albus, Ps. aeruginosa,* and *Ps. fluorescens.* They differ with respect to their relative degree of activity for the aureus strains and also *M. lysodeikticus.* (9) Forty per cent saturation of crude lysates with $(NH_4)_2SO_4$ in the cold precipitates both enzymes, a treatment which also removes active phage from the crude lysate. (10) Both enzymes act synergistically with high speed phage concentrate to cause rapid and premature lysis of viable cells. (11) The following agents inhibit lysis of killed cells by either enzyme in tryptose-phosphate broth: Merthiolate, 0.01%; HCHO, 0.1%; $CuSO_4$, M/1000; $PbCl_2$, M/1000; $HgCl_2$ M/1000; $AgNO_3$, M/1000; and Duponol, 0.01%. Addition of cysteine-HCl, 0.1%, to systems containing $CuSO_4$, merthiolate, $HgCl_2$, and $AgNO_3$ reverses the inhibition. The following chemical inhibitors fail to affect lysis: proflavin, 0.0005%; iodoacetic acid, NaN_3, NaCN, $NaAsO_2$, and semicarbazide — M/1000.

III. *The Virolysin-Phage Lytic System.* A. *Effect of physiological state*: The susceptibility of cells to lysis by the components of phage lysates (pellet and virolysin) depends on their physiological condition, as determined by medium of growth, age upon harvest, medium of suspension, and length of storage.

The degree of lysis effected by any given concentration of either component is related to the age of the freshly harvested test cell as follows: Virolysin alone is unable to lyse rapidly dividing cells. As the culture passes into the resting phase, an increasing number of cells (presumably dying or dead) become susceptible to a given concentration of virolysin. The addition of phage-containing pellet material alone to exponential phase cells causes, or allows to be detected, a significant but small amount of lysis. The degree is directly related to cell susceptibility to spontaneous autolysis. Older cells in the resting phase are completely resistant to pellet material added in large amounts.

The enzyme and pellet act synergistically in producing lysis of viable cells. Exponential phase cells are more susceptible than resting phase cells (Table II). While not indicated in the table, it has been found that the degree of lysis caused by a given amount of pellet and virolysin is directly affected by the tendency of a given age cell to undergo autolysis

TABLE VI. Formation of Virolysin during the Latent Period of Phage 14 Infection of Exponential Phase *S. aureus* K_1 in TP2x Broth.

			Extracellular† material	Intracellular material,‡ removed by lysis of infected cells by mixture of virolysin 14(145) and irradiated P 14(145)			
					Virolysin content		Phage content
Sample	Min. at 37°C*	Turbidity of sample in Klett units	Virolysin released (% lysis 7×10^8/ml heat-killed K_1 cells in 40 min. 37°C)	Lysis of 6×10^8 infected cells/ml after 24 hr 4°C (%)	Lysis of 7×10^8/ml heated cells, 40 min. (%)	Lysis ratio, % infected/ % uninfected	(Plaques/ ml)
Uninfected cells	0	24	0	76	19	1.0	0
Infected "	0	24	0	70	31	1.6	1.3×10^6
	12	24	0	71	34	1.8	1.7×10^7
	24	24	0	86	42	2.2	1.1×10^8
	36	24	0	89	57	3.0	1.1×10^9
(Complete lysis)	50	0	64	—	64	3.4	1.2×10^9

* Latent period of infection = 41 min., as determined by Klett readings of onset of lysis. Infection ratio, P/B = 3/1, initial bacteria = 2.5×10^8/ml, adsorbed in cold for 20 min. prior to start of test.

† Chilled samples were centrifuged at low speed and supernatant layer tested for virolysin activity.

‡ Chilled cells were resuspended into lysing mixture of 1/10 dilution 14(145) virolysin and ultraviolet irradiated phage P 14(145), containing the equivalent of 4×10^{10} irradiated particles/ml. Residual cells and debris were centrifuged off at low speed prior to testing for virolysin and phage.

during storage in the absence of either component.

Lysis of viable cells by combined pellet and virolysin is prevented by the same chemicals inhibiting lysis of heat-killed cells, suggesting that the same enzyme (virolysin) is operative in the lysis of both living and killed cells. Living cells, being resistant to virolysin alone, might contain an inhibiting mechanism to protect them from lysis. It is possible that the addition of virus material functions to destroy or bind the inhibitor so that the cells become susceptible to virolysin. Heat, acetone-extraction, or storage in the cold substitute for the action of the material in phage pellet in that they render the cell susceptible to the enzyme.

Lysis of viable cells by the combined action of pellet and virolysin has been observed in all *S. aureus* strains (25) tested. All strains also adsorb virus, although some do not allow plaque formation.

B. *Evidence for the viral nature of the pellet component* comes from the observation that the degree of lytic activity of a series of different pellet suspensions, prepared from different lots of phage lysates, when tested in combination with a constant amount of virolysin, and a constant number of viable cells, is related to the virus titre by plaque count, Table V. While this suggests that mature phage particles are the active ingredient of the pellet component, the property of infectivity or plaque-forming ability appears unnecessary, for appropriate irradiation with UV light inactivates over 99% of infectivity without destroying lytic activity in conjunction with virolysin, Table V. The evidence to date suggests that intact, adsorbing, but not necessarily infective virus acts synergistically with the extra-virus enzyme, virolysin, to cause a rapid and premature lysis of viable cells with concomitant loss of phage. This phenomenon may be likened to the phenomenon of lysis-from-without, with the qualification that in this particular system virus provides only part of the necessary material.

IV. *Formation and function of virolysin.* Table VI indicates that virolysin is released from virus-infected cells at the end of their latent period at 37°C. When infected cells are broken open prematurely in the cold, significant increases in the enzyme can be detected within them long before the end of the normal latent period, Table VI.

Virolysin probably functions in the release of virus at the end of the latent period, since it is possible to inhibit and reverse lysis at the end of the latent period by treatment with appropriate amounts of $CuSO_4$ (M/750 — M/2000) and cysteine-HCl, 0.1%. $CuSO_4$

TABLE VII. Lysis Inhibition by $CuSO_4$ and Its Removal by Cysteine HCl at the End of the Latent Period of Phage $14(K_1)$ Infection in TP2x Broth.

Treatment of infected* cells (3×10^8/ml)	Onset of lysis at 37°C, min.	Virolysin† released on lysis, % lysis heat killed cells 75 min.	Phage released on lysis/ml
A. Untreated control	35–40	51	1.2×10^9
B. $CuSO_4$ M/750 at 30 min.	>80	1	4×10^7
C. $CuSO_4$ M/750, 30 min. followed by cysteine HCl, 0.1% 35 min.	35–40	14	6×10^8
D. Cysteine HCl, 0.1% 30 min.	35–40	54	1.2×10^9

* Exponential phase cells were infected with $14(K_1)$ phage, P/B = 3/1, in TP2x broth for 15 min. in an ice bath. Samples were then incubated at 37°C and observed for lysis by Klett readings.

† Tests for virolysin were carried out in presence of added cysteine, 0.2% to neutralize $CuSO_4$ inhibition.

added at the end of the latent period inhibits lysis and release of phage (Table VII). This concentration, M/750, does not inhibit cell division in uninfected cells.) Treatment with cysteine-HCl then reverses the inhibition, and lysis and phage release occurs. The $CuSO_4$-cysteine treatment is also effective in preventing and restoring activity of virolysin for heat-killed cells, so that the treatment is effective in blocking and reversing lysis both intra- and extra-cellularly. In the case of intracellular lysis, $CuSO_4$ added at any time during the latent period prevents lysis, but cysteine-HCl reverses the inhibition only at the end of the latent period, about the time when the untreated, infected cells begin to lyse.

The virolysin described above is not peculiar to the phage P14-*S. aureus* K_1 system alone. Similar material is formed during phage P14 infection of another host, *S. aureus* 145, and also by phage P_1 infection of either *S. aureus* K_1 or 145. The exact interrelationship of the various virolysins remains to be determined. Since P_{14} and P_1 are related phages(8), the ability to induce formation of virolysins which function in release of virus and in triggering premature lysis may prove to be a property of these phages, in contrast to other viruses which carry a built-in lytic mechanism (enzyme or enzyme activator) such as is suggested for certain T phages(9).

Summary. Phage infection of staphylococcal cells results in the formation and release of a lytic enzyme which causes the lysis of killed cells, and in conjunction with virus material, lyses living cells. This enzyme appears to be closely related to the autolytic system of uninfected cells, but is different in its pH optimum and activity for *M. lysodeikticus.* It increases during the latent period of phage infection, and we suggest it functions in the ultimate lysis and release of virus from the host cell.

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Immediate Effects of Epinephrine on Phospholipides Turnover and Lipide Partition in Plasma, Liver and Aorta. (21860)

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Investigation has shown that epinephrine has notable effects upon carbohydrate, protein, and lipide metabolism(1-4). This study was undertaken to determine the immediate effects of epinephrine on phospholipide turnover and lipide partition in plasma, liver, and aorta of intact rabbits. Since alterations in lipide metabolism, particularly in the aorta, may be related to the development of arteriosclerosis the effects of chronic injection of epinephrine and other hormones on lipide metabolism and their relationship to the incidence and severity of arteriosclerosis have been investigated(5,6). Dury and DiLuzio(6) found that the amount of P-32 incorporated in the aortic phospholipides of epinephrine-treated rabbits was significantly increased, but this was not observed in rabbits given cortisone for the same period.

In the present study chemical and radioactive-isotope analyses were done on tissues obtained from rabbits 6 hours following subcutaneous injection of epinephrine. It has been shown(7) that the optimum time for the detection of labeled phosphatides in tissues was 6-10 hours after the administration of labeled phosphate.

Materials and methods. New Zealand white female rabbits of pedigreed stock were used. A rabbit pellet diet *without* additives and tap water were available to the rabbits *ad libitum* for several weeks prior to but not the day of experiment. The control animals received a single subcutaneous injection of peanut oil (0.5 ml/kg body weight). The experimental rabbits were administered a single subcutaneous injection of epinephrine in oil (Adrenalin, 1-500; Parke, Davis & Co.) at the dose value of 1.0 mg/kg body weight. Immediately after the administration of the oil or epinephrine 0.5 mc of radioactive phosphate (P-32) was given intravenously. Heart blood samples (4-5 ml) were obtained from all animals at 2, 4, and 6* hours after the P-32 injection for the determination of plasma glucose(8) and radioactive and plasma acid-soluble phosphates(9). After the 6-hour blood sample was obtained the animals were killed with an intravenously administered overdose of EVIPAL (n-methyl-cyclo-hexenyl-methyl barbituric acid) and the whole liver and the aorta were removed. A section of liver (about 0.5 g) was taken for the determination of liver glycogen(10,11). A large specimen of liver, the whole aorta, and a plasma aliquot of the terminal blood sample were submitted to hot alcohol-ethyl ether extractions. The combined alcohol-ether extractions were evaporated under vacuum and the residue redissolved in petroleum ether (b. p. 30-60 degrees). Suitable aliquots of petroleum ether were used for the chemical and radioactive determinations as follows: Total lipide was calculated from weight of residue after evaporation of petroleum ether and drying to constant weight; the lipide phosphorus was determined(12), and the phospholipide values then were calculated from the lipide-P values x 26; total and free cholesterol were determined(13); and neutral fat was calculated by subtracting the sum of the phospholipide, free cholesterol and cholesterol oleate values from the total lipide value. The inorganic and organic acid-soluble phosphates in liver were determined in accordance with the procedure of Zilversmit and DiLuzio(14). Separate liver samples (approx. 2 g) were homogenized in 10% trichloracetic acid. Following neutralization of the filtrate to phenol red and the addition of a magnesia mixture, the samples were kept in the refrigerator overnight to permit the magnesium ammonium phosphate to settle(15). After centrifugation determinations were done for P-31 and P-32 on the supernatant (organic acid-soluble phosphates) and dissolved precipitate (in-

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* At this time 30-40 ml of heart blood was obtained.

TABLE I. Lipide Partitions in Plasma, Liver, and Aorta, Glycemia, and Liver Glycogen in Control and Epinephrine-Injected* Rabbits.

	Controls (7)	Epinephrine (7)	"P" value
	Liver lipides (g %)		
Total lipide	5.23 ± .13 †	6.08 ± .20	.0025
Phospholipide	4.43 ± .14	4.13 ± .22	
Total chol.	.23 ± .01	.24 ± .01	
Free "	.22 ± .009	.21 ± .01	
Ester "	.01 ± .008	.03 ± .004	.05
Neutral fat	.59 ± .12	1.71 ± .16	<.001
	Plasma lipides (g %)		
Total lipide	.3497 ± .0304	.3496 ± .0360	
Phospholipide	.0949 ± .0044	.1217 ± .0089	.018
Total chol.	.0552 ± .0055	.0718 ± .0103	
Free "	.0184 ± .0020	.0228 ± .0028	
Ester "	.0368 ± .0035	.0490 ± .0076	
Neutral fat	.1627 ± .0154	.1653 ± .0140	
	Aorta lipides (g %)		
Total lipide	8.17 ± .64	9.00 ± .64	
Phospholipide	.944 ± .038	.998 ± .091	
Total chol.	.12 ± .005	.12 ± .006	
Free "	.10 ± .007	.10 ± .005	
Ester "	.02 ± .007	.02 ± .004	
Neutral fat	7.08 ± .66	7.81 ± .64	
Plasma glucose, mg %	127.4 ±2.6	500§	<.001
Liver glycogen, g %	3.07± .47	.205 ± .092	<.001
Rabbits wt, kg	2.4 (1.8–2.7)‡	2.5 (2.0–3.2)	
Liver wt, g/kg	28.8 (23.9–38.2)	29.3 (23.4–39.8)	
Whole aorta, g	.460 (.345–.606)	.498 (.344–.654)	

* Animals were sacrificed 6 hr after epinephrine, s.c. † Mean ± S.E. ‡ Mean and range for group. § All values were 500 mg % or greater.

organic acid-soluble phosphates). For the determination of phospholipide or acid-soluble P-32 suitable aliquots were mounted on Whatman ash-free filter paper backed by aluminum foil, and the samples allowed to evaporate. After covering the sample with Scotch tape, it was counted with a Victoreen No. 1B85 Geiger tube.

Results. The average concentrations of the various lipide constituents of plasma, liver, and aorta of control and epinephrine-injected rabbits are shown in Table I. The liver total lipide, esterified cholesterol, and neutral fat concentrations were significantly elevated in the epinephrine-treated rabbits. With regard to the plasma lipide partition, only the phospholipides were significantly increased. The concentrations of the various aortic lipide fractions in the two groups of rabbits were not different. The influences of epinephrine upon carbohydrate metabolism in the rabbits of this study are evident from the high blood glucose and low liver glycogen concentrations found in the experimental animals.

Several of the above-mentioned observations are in accord with the experience of other investigators when epinephrine was injected into man or experimental animals. It is well-known that the hyperglycemia which follows the injection of epinephrine is effected by an increase in liver glycogenolysis, and probably a decrease in peripheral utilization of glucose. An increase in liver fatty acids and phospholipides was observed by Pollack

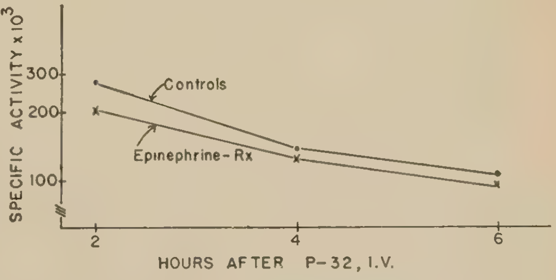

FIG. 1. Semilogarithmic plot of avg specific activity of plasma acid-soluble phosphate at 2, 4, and 6 hr after intravenous P-32 administration. Means were derived from data on 7 control and 7 epinephrine-injected rabbits.

TABLE II. Effect of Epinephrine on Lipid and Acid-Soluble Phosphates Turnover in Rabbits.

	Controls (7)	Epinephrine* (7)	"P" value
Phospholipide-P S. A. × 10^3			
Liver	10.1 ± 1.3†	14.5 ± 1.0	.017
Plasma	4.3 ± .4	6.7 ± .3	<.001
Aorta	4.8 ± .6	11.1 ± 1.2	<.001
Inorganic-P S. A. × 10^3			
Plasma	108.3 ± 5.7	97.1 ± 5.2	
Liver	113.2 ± 19.1	111.6 ± 4.5	
Organic ac. sol.-P S. A. × 10^3			
Liver	45.9 ± 6.7	42.6 ± 2.9	

* Animals sacrificed 6 hr after subcut. epinephrine inj.

† Mean ± S.E.

S. A. = Specific activity expressed as the percentage of the inj. P-32/mg phosphorus.

(16) 2-3 hours after epinephrine injection in rabbits.

In Fig. 1 are presented the specific activities of the plasma acid-soluble phosphates at 2, 4, and 6 hours after P-32 administration in control and epinephrine-injected rabbits. It is evident that the specific activity curves of the two groups were not different. Thus, the epinephrine administration probably had not influenced the turnover of the acid-soluble phosphates. It therefore is unlikely that the latter had any appreciable effect on the phospholipides turnover in the tissues which were studied.

In Table II are presented the average specific activities calculated as the percentage of the administered P-32 in the phospholipides fraction or the acid-soluble phosphates per mg of phosphorus. These data show that the specific activities of the phospholipides of liver, plasma, and aorta were significantly greater in the epinephrine-treated rabbits than the respective control values. The specific activities of the terminal plasma acid-soluble phosphates, and the liver inorganic and organic acid-soluble phosphates were not different in the two groups of animals. Thus, the increased turnover of liver phospholipides in the epinephrine-treated rabbits cannot be attributed to an altered rate of P-32 incorporation in the precursors of liver phospholipides. Since it is believed that plasma phospholipides are derived mainly from the liver (17), the results indicate that the synthesis of liver and plasma phospholipides was accelerated in the rabbits injected with epinephrine.

Discussion. Particularly interesting is the observation that the aortic phospholipides specific activity was significantly increased following epinephrine administration. The results in fact show that the rate of aortic phospholipide synthesis was greater by 131% in the epinephrine group compared with the controls while there was a 43% increase in phospholipide turnover in the liver, which normally is the most active phospholipide synthesizing tissue in the body. That the aorta is capable of synthesizing phospholipides *in vitro* was shown by Chernick *et al.*(18) in studies of the normal rat aorta. The importance of the present results is that following epinephrine administration the rate of phospholipide formation was accelerated in liver and to an even greater extent in arterial tissue, which is believed to be a very slowly metabolizing tissue. In all instances the data showed that the specific activity of the aortic phospholipides in the epinephrine-injected rabbits was greater than the average or the individual terminal plasma phospholipides specific activity. It seems improbable from the present evidence that special plasma phospholipides of high specific activity had been selectively incorporated into the aorta from the plasma during the 6-hour period since the rabbits were injected with epinephrine. The results therefore strongly indicate that an increased rate of aortic phospholipide synthesis was induced in the treated rabbits.

The physiological significance of the increased phospholipides metabolism in the liver and aorta of the epinephrine-treated rabbits is not evident from the results of this study. There is evidence(19) of a relationship between the rate of phospholipides synthesis and the metabolic needs of the tissue, such as an increase in the rate of fat oxidation. It is generally believed that one of the prominent actions of epinephrine is to increase the rate of fat catabolism(20). However, only the actual demonstration that epinephrine changed the rate of fat metabolism and the formation of phospholipides in these tissues would be acceptable as evidence of this relationship. The present findings indicate that one of the modes of epinephrine influence on lipide metabolism was an increased rate of phospholipides formation in liver and aorta. Whether this observation is in any way related to the effect of epinephrine in producing injury to the medial wall of the aorta must await further studies.

Summary. 1. The lipides partition and phospholipides turnover in plasma, liver, and aorta of intact rabbits was determined 6 hours after the subcutaneous injection of 1 mg/kg epinephrine in oil. 2. Significant changes were found in certain lipide fractions of liver and plasma but no alterations in the concentrations of the various aortic lipide fractions. 3. Specific activities of the plasma, liver and aortic phospholipides were significantly elevated in the rabbits given epinephrine; a particularly large change was found in the rate of aortic phospholipides formation. The possible relationship of the latter finding to the development of arteriosclerosis was discussed.

The technical assistance of Frank Szematowicz and Janice Stoneking is gratefully acknowledged.

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Influence of Calcium and Serum Albumin on Clearing of Lipemic Dog Plasma *In vitro*. (21861)

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The clearing *in vitro* of lipemic plasma by heparin clearing factor(1) has been studied in a variety of systems and the effects of temperature(2), pH(3,4), salt concentration(3), dilution(5,6), dose of heparin(5-7) and substrate(6) are well recognized. Since there were no studies reported on the effects of anticoagulants we investigated the rate and degree of clearing in 3 different anticoagulant systems.

Materials and methods. Lipemic plasma (LP) obtained from dogs administered 20-25 ml cottonseed oil/kg intragastrically was considered satisfactory if optical density did not exceed 0.06. Plasma containing clearing factor(CF) was obtained from dogs 30 minutes after intravenous administration of 4 mg heparin Na/kg. All plasma was separated from freshly drawn blood, centrifuged at 4°C and used immediately. Coagulation was prevented by collecting the blood in either a siliconed system or by adding anticoagulants. The siliconed system was prepared by the method of Jaques(8). Solutions of the following anticoagulants in physiological salt solution (PSS) were diluted 1:9 with whole blood: 0.1 M sodium citrate, 2% disodium ethylenediaminetetraacetate (EDTA) and 1% Manuronate®*. Defibrination of heparinized plasma was accomplished by shaking vigorously with glass beads for 5 hours at 4°C and centrifuging at 2000 rpm. *Decalcification* by the use of Amberlite IR-100(9) could not be done because the resin was not available. The manufacturer's recommendation of substituting Amberlite IR-120 was followed. Columns containing 50 g of IR-120 kept at 4°C were washed with 50 ml demineralized water and allowed to drain completely to remove all color. The effluents obtained by passing 10 ml quantities of plasma through each column were analyzed for total protein N by the micro-Kjeldahl method and for lipid P by the method of Fiske and SubbaRow(10). The values were compared with those obtained on aliquots of the original plasma and of the eluate following discharge by 10 ml of 0.1 N HCl. After passage of CF the resin was layered over dry $NaHCO_3$ to protect the retained CF from long contact with the acid used in discharging. The method of Roe and Kahn(11) was used on plasma as a qualitative demonstration of the relative capacities of citrate and EDTA to bind Ca and prevent its precipitation by phosphate. Lipemia clearing was carried out at 22°C by the method previously described(12) except that CF plasma was diluted 1:4 with PSS. Optical density (OD) was measured at 650 mu in a Beckman model B spectrophotometer adapted to use silica micro-cuvettes (13). Standardization was against distilled water.

Results. Fig. 1 demonstrates the slowing of clearing caused by dilution of CF with PSS. A dilution of 1:4 CF was chosen for the studies shown in Table I to demonstrate the effect of various anticoagulants on the clearing of LP by diluted CF. The degree and rate of clearing were greater and faster in the siliconed system than in the others. Turbidity due to formation of soaps did not occur during the 2 hours of observation. EDTA bound considerably more Ca than did citrate and was less effective in maintaining activity of CF. Manuronate or defibrination which affect plasma proteins instead of binding Ca also retarded clearing. A sample of heparin CF which reduced optical density from the initial 0.80 to 0.15 lost all activity by passage through IR-120. Addition of $CaCl_2$ (18 mg% Ca) to the CF eluate restored approximately 75% of the original activity. CF discharged from the column by HCl and neutralized with $NaHCO_3$ had approximately 80% of the initial clearing activity. Passage of CF through IR-120 resulted in complete removal of Ca and lowering of total protein N from 11.9% to 0.12%. Passage of LP through the column resulted in a similar decrease of total protein N and a decrease of lipid P from the initial 11 mg% to 0.81 mg%. HCl discharge of the LP column resulted in partial recovery of N and P in the eluate.

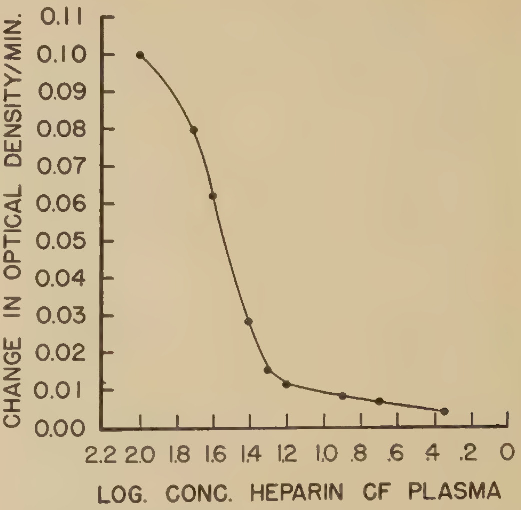

FIG. 1. Effect of diluted heparin clearing factor plasma added to lipemic plasma on the rate of clearing.

Since the preceding studies appeared to demonstrate that Ca and protein were essential for maintaining the optimum rate of lipemia clearing by heparin CF the following experiments were made on separated chylo-

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* A synthetic heparinoid—Wyeth Laboratories.

TABLE I. Effect of Anticoagulants on Lipemia Clearing *in Vitro.*

LP collected in	CF collected in	Optical density after min. of incubation										
		0	5	10	15	30	45	60	75	90	105	120
Siliconed system	Siliconed system	1.0	1.0	.88	.82	.65	.55	.47	.33	.27	.24	—
	Citrate	1.0	1.0	.96	.92	.75	.66	.55	.47	.38	.32	—
	EDTA	1.0	1.0	1.0	1.0	1.0	1.0	.85	.75	.62	.52	—
Citrate	Citrate	.72	.72	.72	.69	.60	.53	.40	.36	.33	.31	.22
	EDTA	.72	.72	.72	.72	.72	.72	.69	.67	.65	.62	.58
	Manuronate	.72	.72	.72	.72	.68	.65	.60	.53	.53	.50	.44
EDTA	Citrate	.79	.79	.79	.74	.72	.67	.60	.56	.52	.50	.46
	EDTA	.79	.79	.79	.79	.79	.77	.74	.67	.63	.56	.55
	Manuronate	.79	.79	.79	.74	.72	.69	.67	.65	.63	.63	.63
Manuronate	Citrate	.74	.74	.74	.72	.62	.58	.52	.49	.42	.39	.34
	EDTA	.74	.74	.74	.74	.74	.74	.72	.69	.65	.62	.55
	Manuronate	.74	.74	.74	.72	.69	.65	.55	.52	.49	.44	.39
Citrate	Citrate	.74	.74	.72	.69	.67	.62	.52	.45	.38	.34	.30
	Amberlite	.74	.74	.74	.74	.74	.74	.74	.74	.74	.74	.74
	Defibrinated	.74	.74	.74	.74	.72	.65	.60	.54	.50	.44	.41
Citrate	Amberlite	1.39	1.39	1.39	1.39	1.39	1.39	1.39	1.39	1.39	1.39	1.39
	" + 9 mg % Ca	1.39	1.39	1.39	1.39	1.30	1.09	.95	.82	.72	.62	.58
	" + 18 " "	1.39	1.39	1.39	1.39	1.30	.85	.74	.60	.52	.45	.40

micra. 40 cc of citrated LP were centrifuged at 11,000 rpm in a Servall refrigerated centrifuge at 4°C for 30 minutes. The supernatant was decanted and the settled chylomicra were divided into approximately 6 equal portions and suspended in the following in a concentration to approximate the optical density of the original plasma: PSS, PSS + 18 mg% Ca as $CaCl_2$, PSS + 7.5 mg% Ca, PSS + 2% human serum albumin, PSS + 2% human serum albumin + 7.5 mg% Ca, and non-lipemic citrated dog plasma. The suspensions were tested for clearing by CF and the rate compared with that of the original LP. The rates[†] of clearing in optical density units per minute were as follows: LP 0.5, chylomicra in normal plasma 0.45, and chylomicra in PSS 0.01. Addition of 7.5 mg% Ca to the suspension in PSS increased the rate to 0.20 but greater concentrations of Ca resulted in agglutination of the chylomicra, increased optical density and failure to be cleared by heparin CF. The addition of serum albumin to chylomicra in PSS also increased the rate of clearing (0.28) while the addition of both albumin and Ca restored the rate (0.39) to nearly that of LP.

Discussion. The rate of clearing observed by us for CF released after intravenously administered heparin agrees with that reported by French *et al.*(7) and Grossman(6) and also confirms that diluting CF(7,14) slows optical clearing. *In vitro* dilution (Fig. 1) has an effect comparable to injecting smaller doses of heparin. It has been reported that EDTA retards the clearing of lipemic plasma *in vitro*(15). Our results confirm this observation and indicate that the slowing is due to binding Ca. Change in optical density of LP by heparin CF is associated with the release of free fatty acids and is linear with respect to time until sufficient soap formation occurs to cause returbidification(16). Ca and serum albumin are the fatty acid acceptors necessary to maintain the reaction(17,18) and accumulation of fatty acids acts as an inhibitor to clearing(19). It has also been reported that purified CF does not clear chylomicra suspended in PSS unless plasma is added(14). Our findings are consistent with all these views but emphasize the need for standardization of the system used to estimate lipemia clearing. This is obvious from our data which show that varying the concentration of either acceptor alters the rate of clearing.

Summary. 1. Studies are reported on the effect of 3 different anticoagulant systems on the rate and degree of optical clearing of lipemic plasma by heparin clearing factor plasma. 2. Calcium binding anticoagulants decreased lipemia clearing proportional to the binding. 3. Protein binding anticoagulants also retarded or abolished clearing. 4. Chylomicra suspended in normal plasma cleared at a faster rate than those suspended in physiological salt solution. The addition of Ca or albumin enhanced the rate of clearing in physiological salt solution and the addition of both Ca and albumin resulted in almost complete restoration of clearing activity.

[†] Rate of clearing $= \dfrac{\text{Initial OD} - \text{Final OD}}{\text{Min. of incubation}}$.

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Observations on Role of Free Sulfhydryl Groups in Prothrombin Activation.*† (21862)

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Sulfhydryl-containing substances have been reported to cause inhibition of blood coagulation *in vitro* (for references see(1)). The available information concerning the mechanism of this inhibition and the particular stage or stages of the over-all system involved has been limited. However, recently Carter and Warner have indicated that the inhibitory action of SH-containing substances may be due to their ability to reduce essential disulfide linkages in the prothrombin molecule and thereby decrease its reactivity(1,2).

We have observed that the addition of reduced glutathione to normal human plasma is followed by an immediate decrease in the non-protein sulfhydryl concentration of the mixture over and above that accountable for by reaction with prothrombin alone, and a concomitant increase in the non-protein disulfide level probably indicating formation of oxidized glutathione(3). It occurred to us that SH-containing substances might inhibit clotting not only by reducing essential disulfide linkages in prothrombin, but also by giving rise to disulfide substances which might then act as inhibitors by oxidizing essential

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* Some of our results were presented before the 39th Annual Meeting of Fed. of Am. Soc. for Exp. Biol., San Francisco, Cal., April, 1955.

† Supported by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army.

sulfhydryl groups present in other clotting factors. It seemed proper, therefore, to examine the effects on the clotting mechanism of both the reduced and oxidized forms of a given sulfhydryl substance. Glutathione was selected for this purpose as it is present in most animal tissues and both forms of this substance are readily available and soluble.

Methods and materials. Samples of blood were collected by venepuncture using the 2-syringe technic. Nine parts of blood were mixed with one part of 3.2% sodium citrate, the plasma was separated by centrifugation and used experimentally within 2 hours following collection. Only plasmas having normal prothrombin levels as determined by the 2-stage technic(4) were used. One-stage prothrombin assays were done as described by Quick *et al.*(5) using Soluplastin (Schiefflin) as a source of thromboplastin and M/40 calcium chloride. Two-stage prothrombin assays were carried out using the modified technic of Ware and Seegers(4). The pre-incubation system was essentially the same as that used by Carter and Warner(1) and consisted of 0.30 ml 0.03 M Veronal buffer (pH 7.05), 0.20 ml of the reagent under investigation and 0.10 ml defibrinated plasma or purified prothrombin. Unless otherwise specified, pre-incubation was allowed to proceed for 60 minutes at room temperature before the determination of the thrombin yield by the modified 2-stage procedure. Control experiments were done in which 0.85% saline was added instead of reagent and the results obtained were expressed in terms of percentage inhibition as related to the controls. All experiments were carried out at least in triplicate and the data reported are the averages. Sulfhydryl determinations were done by an amperometric procedure(6). The disulfide levels were measured as described by Carter(7). Purified prothrombin, reduced and oxidized glutathione as well as all other SH-oxidizing and blocking agents were made up in twice glass-distilled deionized water and adjusted to pH 7.10 immediately prior to the start of each experiment. Reduced glutathione (GSH), oxidized glutathione (GSSG) and N-ethyl maleimide were obtained from Schwarz Laboratories, Inc., Na-iodoacetate, alloxan and 1, 2-naphthoquinone-4-sulfonic acid Na-salt were Eastman Kodak products, p-chloromercuribenzoic acid, p-chlorophenylsulfonic acid and iodosobenzoic acid were obtained from Sigma Chemical Co., Salyrgan from Winthrop-Stearns, Inc., and phenyl mercuric hydroxide from Matheson, Coleman & Bell, Inc. Na-tetrathionate was provided through the courtesy of Dr. I. C. Winter of G. D. Searle & Co. All other chemicals used were of standard reagent grade.

Effects of GSH and GSSG on One-Stage Prothrombin Times of Normal Human Plasma

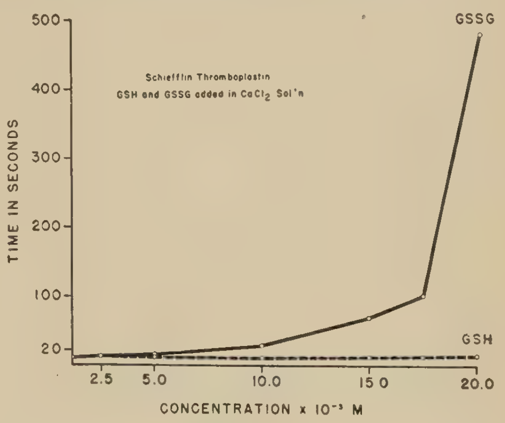


FIG. 1.

Results. Effects of GSH and GSSG in One-Stage System. The effects of reduced and oxidized glutathione were first examined in the usual 1-stage prothrombin assay system, the reagent to be tested being added in the calcium chloride solution. GSH up to a

Effect of Simultaneous Presence of GSH and GSSG on One-Stage Prothrombin Times

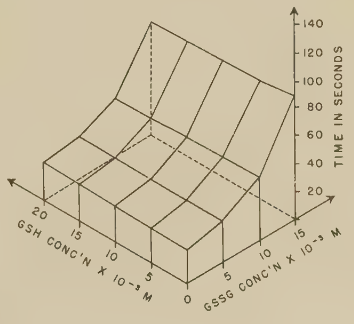
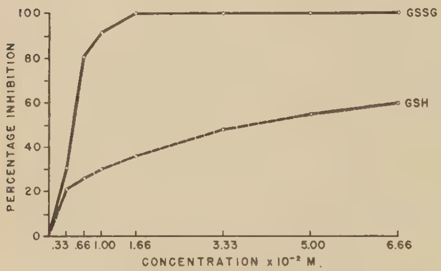


FIG. 2.



Changes in Two-Stage Prothrombin Values Resulting From Pre-incubation of Human Plasma with GSH or GSSG

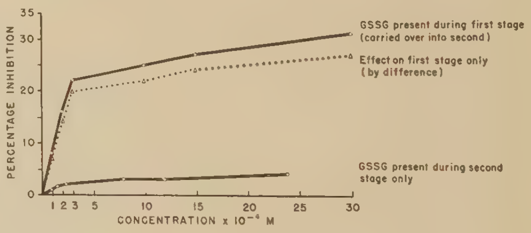
FIG. 3.

concentration of at least 20 x 10^{-3} M produces practically no inhibition whereas GSSG doubles the normal prothrombin time in a concentration of 10 x 10^{-3} M. (Fig. 1).

On the assumption that the GSSG effect may be due to the oxidation of essential SH groups on one or more clotting factors it seemed of interest to determine whether the simultaneous presence of GSH in concentrations having no effect on the 1-stage results might decrease the inhibition obtained with GSSG alone. Both reagents were made up in the calcium chloride solution in different concentration ratios and then added to the 1-stage system. Over the range of concentrations used, the additional presence of GSH was without effect and the resulting inhibitions were of the same order of magnitude as those observed when the respective level of GSSG alone was present (Fig. 2). It would appear that an increased disulfide concentration inhibits either thrombin formation or fibrinogen clotting or both, whereas an increased SH level, at least up to a concentration of 20 x 10^{-3} M, has no significant effect at these points in the clotting mechanism.

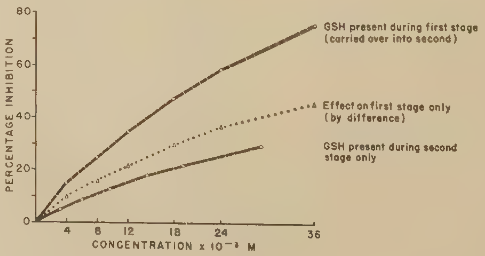
Effects of GSH and GSSG in Two-Stage System. Next, prothrombin activation was studied in a 2-stage system following pre-incubation of plasma with GSH and with GSSG. It is apparent (Fig. 3) that with increasing concentrations GSH causes increasing inhibition of prothrombin activation and that levels which are without effect in the 1-stage system will produce significant effects when allowed to incubate with plasma prior to the activation of prothrombin. GSSG, on the other hand, in concentrations of 0.5 x 10^{-2} M and higher will bring about much more marked inhibitions of thrombin formation than does GSH.

Since in the determination of prothrombin any glutathione present during the pre-incubation period is carried over into the first and second stages of the 2-stage assay and may exert effects at one or both of these levels, experiments were done to determine what part, if any, of the total inhibition observed is contributed by interference with prothrombin activation of fibrin formation. To obtain the combined effects on the first and second stages the reagent to be tested was made up to the appropriate concentrations in the diluent and added at the start of the 2-stage assay. The effect on the second stage only was obtained by adding the reagent in the fibrinogen. The results of these experiments carried out with GSSG are presented in Fig. 4. The top curve represents the total inhibition observed when GSSG is added at the start of the first stage and then carried over into the second, whereas the lower curve indicates the inhibition



Plasma Prothrombin Determination as Affected by Addition of GSSG to First or Second Stages of Two-Stage

FIG. 4.



Plasma Prothrombin Determination as Affected by Addition of GSH to First or Second Stages of Two-Stage

FIG. 5.

TABLE I. Summary of Data. Two-stage plasma prothrombin values as affected by presence of GSH or GSSG during different stages of the experiment.

	Reagent added at beginning of:		
	60 min. pre-incubation stage	First stage of two-stage	Second stage of two-stage
Reagent dilution	1:3	1:60	1:75
GSH conc. at various stages	6.66×10^{-2} M	3.33×10^{-3} M	2.66×10^{-3} M
% inhibition	60	8	4
GSSG conc. at various stages	0.66×10^{-2} M	0.33×10^{-3} M	0.26×10^{-3} M
% inhibition	81	20	2

found when it is present during the second stage only. The dotted curve, on the other hand, is obtained by difference and indicates the inhibition due to interference with prothrombin activation. As is apparent from the data the over-all inhibition observed in the presence of GSSG is primarily due to a decrease in prothrombin activation.

The corresponding data for GSH (Fig. 5) indicate that with this substance a relatively greater part of the total inhibition is due to interference with fibrin formation.

Table I indicates how these data can be used to obtain the contributions to the over-all inhibition of each of the 3 experimental phases, *i.e.* pre-incubation, first and second stages. From a calculation of the differences between the inhibitions observed when either form of glutathione is added at the start of any of the 3 experimental stages it is apparent that for both forms of glutathione by far the largest part of the overall effect observed is due to changes in one or more clotting factors taking place prior to the start of the 2-stage assay. Of additional interest is the fact that, as in the 1-stage system, the same degree of inhibition is brought about by considerably lower concentrations of GSSG than of GSH.

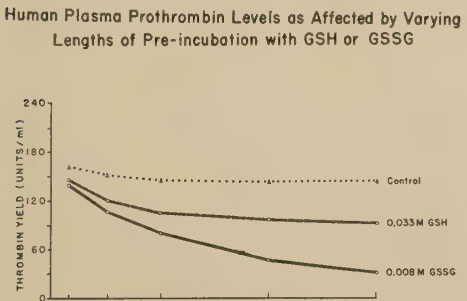


FIG. 6.

If, as it appears, the 2 forms of glutathione react with certain clotting factors present in plasma one might expect that as the pre-incubation period is lengthened the resulting inhibitions would become more pronounced. The results of such an experiment are presented in Fig. 6. They show that the thrombin yield decreases progressively for both GSH and GSSG as the pre-incubation time with plasma is increased from 5 to 90 minutes and GSSG was again found to exert the more pronounced effect.

Working with purified bovine prothrombin Carter and Warner have presented evidence to suggest that the inhibitory effect of GSH may be due to its reaction with prothrombin disulfide linkages(2). Their postulate is well supported by our data. In addition, we have observed that pre-incubation of purified bovine prothrombin‡ with GSSG also results in decreased thrombin formation. The effect of GSSG is rather more marked than that of GSH and very similar quantitatively to the one obtained following pre-incubation with human plasma. (Fig. 7).

Effects of SH-blocking Agents in Two-Stage System. The inhibition produced by GSSG is difficult to explain on the basis of an interaction with prothrombin sulfhydryl groups since it has not been possible to detect any such groups in this molecule(2,3). Support for an hypothesis involving different loci of action for GSH and GSSG has come from experiments in which human plasma was pre-incubated with GSH and various SH-blocking

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‡ Kindly supplied to us by Dr. Walter H. Seegers, Wayne University Medical School, Detroit, Mich. Lot No. 541002.

Changes in Two-Stage Prothrombin Values Resulting From Pre-incubation of Purified Prothrombin with GSH or GSSG

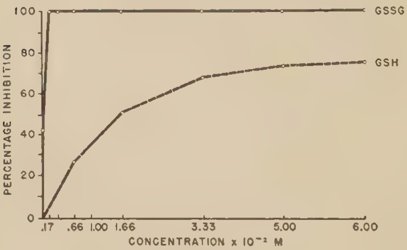

FIG. 7.

agents simultaneously. We not only find greater inhibition under these conditions than in the presence of GSH alone, but, contrary to the findings of Carter and Warner with dog plasma(1) and purified bovine prothrombin(2), the SH-blocking agents by themselves also result in inhibitions (Fig. 8). Furthermore, for any given combination, the total inhibition observed is approximately equal to the sum of the individual inhibitions obtained with GSH and the particular SH-blocking agent used. Similar inhibitions of thrombin formation in human plasma have been observed with N-ethyl maleimide, Salyrgan, p-chloromercuribenzoic acid and p-chloromercurisulfonic acid. As in the case of the 2 forms of glutathione the major part of the inhibitions observed with the SH-blocking agents appears to be due to reactions taking place during the pre-incubation period. Experiments are now under way to determine whether these substances produce similar inhibitions of thrombin formation following pre-incubation with purified bovine prothrombin. The absence of such an inhibition in the presence of some SH-blocking agents has been reported by Carter and Warner(2).

Changes in Two-Stage Prothrombin Values Resulting From Simultaneous Pre-incubation of Human Plasma with GSH and -SH-blocking Agent

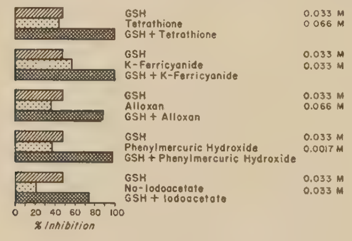

FIG. 8.

Since the difference between the observations made by these workers with dog plasma and those with human plasma reported here might be due to differences in the relative concentrations of one or more accelerators in the plasmas of these two species, experiments were carried out with various SH-oxidizing agents and both human and dog plasmas. For every one of the reagents tested the degree of inhibition obtained was found to be considerably less with dog plasma than with human plasma, the most striking difference being that observed with GSSG (Fig. 9). The additional finding that increasing GSSG concentrations in the presence of dog plasma result in inhibitions more nearly of the order of those found with human plasma provides additional support for the idea that GSSG reacts with a prothrombin conversion accelerator which is present in higher concentrations in dog plasma than in human plasma (Fig. 10).

Changes in Two-Stage Prothrombin Values Resulting From Pre-incubation of Human and Dog Plasmas with -SH-oxidizing Agents

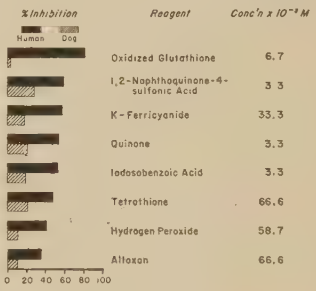

FIG. 9.

Changes in Two-Stage Prothrombin Values Resulting From Pre-incubation of Human or Dog Plasma with GSSG

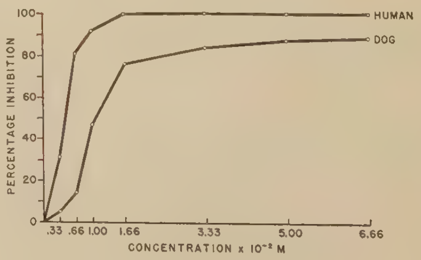

FIG. 10.

Discussion. If it can be assumed that the lack of detectable SH groups in purified bovine prothrombin is indicative of their actual absence in this molecule, then the inhibitions produced by GSSG and various other SH-blocking agents would have to be explained on the basis of an interaction with a clotting factor other than prothrombin. However, it is difficult definitely to exclude the possibility that prothrombin itself may somehow be involved. Thus, it could be that SH groups are actually present in this protein although inaccessible to the silver ions used in the amperometric titration, or that prothrombin in plasma does contain free SH groups which, however, during the process of purification have become oxidized to disulfide linkages. However, purified bovine prothrombin containing disulfide linkages but no detectable SH groups can nevertheless be readily activated to yield thrombin. On the other hand, if free SH groups are present, although not demonstrable chemically, addition of purified prothrombin to the plasma pre-incubation system should decrease the inhibitions caused by SH-oxidizing and -blocking agents. However, this is not found to be the case(3). From the above it would appear, therefore, as rather unlikely that prothrombin itself plays a direct part in the observed inhibitions.

In the plasma experiments by far the largest part of the total inhibition found appears to be due to reactions occurring during the pre-incubation period, indicating that the factor or factors involved possess accelerator properties in the presence of tissue thromboplastin and Ac-globulin (both of which are added in the 2-stage assay). Among these could be any one or several of the following: Antihemophilic factor (AHF) (platelet cofactor I of Seegers and associates(10)), the plasma precursor of factor VII, plasma thromboplastin component (PTC) of Aggeler and associates(8) (platelet cofactor II of Seegers and co-workers(11)), or plasma thromboplastin antecedent (PTA) of Rosenthal *et al.* (9). However, with GSSG the same degree of inhibition is also observed when purified prothrombin is substituted for plasma, seeming to eliminate AHF, PTC and PTA providing it can be assumed that none of these materials is present in highly purified bovine prothrombin preparations. Under these conditions the observed inhibitions might be explained by postulating the occurrence of a pre-incubation reaction with a prothrombin derivative formed in solution and prior to activation by thromboplastin and Ac-globulin. This could be the autoprothrombin described by Seegers which he has shown to be derived from prothrombin and to possess factor VII-like characteristics(12). It may also be possible that even highly purified bovine prothrombin may contain small amounts of platelet cofactor II (PTC)(13) and this might provide an alternative explanation of the GSSG inhibitions found with purified prothrombin.

Future experiments may enable us to state with more certainty which of the various possible factors is actually involved in the observed inhibitions. With prothrombin requiring disulfide linkages for activity and some still undefined conversion accelerator requiring free SH groups for its functioning it would appear that physiological and chemical conditions affecting the oxidation or reduction of these groups may influence indirectly the rate of thrombin formation and thereby possibly even determine the occurrence of fibrinogen clotting. Thus, it may be that a compensatory mechanism exists in blood which over a wide range of conditions affecting sulfhydryl-disulfide interrelations tends to maintain the rate of thrombin formation within certain "normal" limits. Only extreme fluctuations in some of these conditions would tax the compensatory system beyond its capacity and result either in little or no thrombin formation possibly resulting in hemorrhagic complications or excessively rapid thrombin formation and intravascular clotting.

Summary. (1) One-stage prothrombin determinations in human plasma are only slightly affected by a wide range of GSH concentrations whereas relatively low levels of GSSG result in a marked lengthening of the prothrombin times. (2) Incubation of human plasma with either GSH or GSSG prior to initiation of prothrombin activation produces inhibitions of thrombin formation, the same degree of inhibition being observed with

considerably lower concentrations of GSSG than of GSH. (3) A study of the precise point of action of the two forms of glutathione reveals that at least 75% of their over-all effects are due to reactions with clotting factors occurring during the pre-incubation period. (4) Pre-incubation of human plasma has resulted in an immediate decrease in the non-protein sulfhydryl concentration of the mixture over and above that accountable for by reaction with prothrombin. A concomitant increase in the non-protein disulfide level of the system appears to indicate the formation of some GSSG and supports the view that GSH inhibits clotting not only by reducing prothrombin disulfide linkages, but also by giving rise to some GSSG which, as has been shown subsequently, has marked inhibitory powers of its own. (5) Data have been presented which support the hypothesis that GSSG as well as other SH-oxidizing and -blocking agents react with at least one prothrombin conversion accelerator which is present in higher concentrations in dog plasma than in human plasma. This, in turn, implies the essential nature of free SH groups on such an accelerator and establishes their importance in the blood clotting mechanism. (6) Possible explanations and implications of these findings have been discussed.

We are pleased to acknowledge the valuable technical assistance of Miss Lillian V. Novak.

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Dietary Necrotic Liver Degeneration and Coenzyme A. (21863)

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Dietary necrotic liver degeneration is produced in rats by a 30% *Torula* yeast diet restricted in cystine and free of vit. E and Factor 3(1,2). Animals on such rations develop a metabolic lesion in the liver several weeks before pathological changes become evident. This defect is characterized by a marked decline in oxygen consumption of liver slices after an initial period of normal respiration (3,4). The 3 factors protecting against dietary liver necrosis also prevent the respiratory decline. Respiratory decline is accompanied by an impaired utilization of acetate for lipogenesis, ketogenesis, and oxidation to CO_2, which appears to be secondary to the primary defect in oxidative metabolism(5).

Olson and collaborators reported that the CoA contents of prenecrotic livers from animals on soybean protein rations are reduced. They stated that an etiological relationship of reduced CoA synthesis to hepatic necrosis is possible(6), and that vit. E prevents, to a large extent, the changes in CoA contents in these animals(7). We have investigated liver CoA levels in rats fed the necrogenic *Torula* yeast diet, and have tested the effects on liver

TABLE I. Relationship between Severity of Pathological Lesion of Dietary Necrotic Liver Degeneration and CoA Content of Livers of Rats Fed the Basal Diet.*

No. of rats	Final body wt, g	Gross appearance of liver	Liver Sample taken for assay†	Liver CoA content,‡ μM/g
7	88	Normal	"normal"	.24 ± .02
7	68	Small areas of necrosis or congestion	"	.23 ± .05
4	75	Clearly demarcated areas of necrosis, some involving all of a smaller lobe	"	.21 ± .04
5	83	Extensive and severe necrosis	necrotic	.07 ± .06

* Rats were fed the basal *Torula* diet for 30-50 days.
† Respiration of slices declined more than 25% during 2 hr incubation.
‡ Mean and stand. dev.

CoA values of various factors that protect against dietary necrotic liver degeneration. The liver CoA contents of animals on the basal *Torula* yeast diet were found to be greatly reduced. However, vit. E, and also Factor 3, failed to influence liver CoA values, and no correlation was found between CoA levels and the incidence of necrosis or of the respiratory decline.

Procedures. Details of procedures applied have been published(1,2,4). In the present study, weanling, male Sprague-Dawley rats of the National Institutes of Health strain were fed the basal 30% *Torula* yeast diet*† for 21 days. Subsequently, some groups were continued on this diet, whereas others were transferred to rations consisting of the basal diet plus one of the following supplements: (a) 50 mg% α-tocopherol acetate. (b) 4% of a commercial Brewer's yeast autolysate (64 units Factor 3/100 g diet(8)). (c) 1% crude tissue powder (59 units Factor 3/100 g diet). (d) Crude Factor 3 preparation from tissue powder (29 units Factor 3/100 g diet). (e) 1% L-cystine. (f) 1.23% DL-methionine. (g) 1% L-cysteine.

One group was *injected intraperitoneally* with 1 mg calcium pantothenate for 4 consecutive days. *Normal controls* were fed a commercial stock diet. Rats were sacrificed at intervals and pieces of liver were taken for determination of the CoA content, and for the measurement of oxygen consumption and of respiratory decline by slices in the Warburg. Slices were incubated in the oxygenated Krebs-Ringer-phosphate buffer with 0.01 M added glucose(4). The temperature was 37.5° and the gas phase was O_2. The decline in oxygen uptake from the initial 30-minute value to that found in the 90-120 minute period of incubation is reported as % of the initial rate. *CoA contents* were determined by the methods of Kaplan and Lipmann(9) and of Stadtman(10). The sample of liver was homogenized in the cold with 9 volumes of water, and enzymes were then inactivated by placing the homogenization tube in a boiling water bath for 4 to 5 minutes. The tube was chilled, centrifuged, and the supernatant stored at −15°C until analyzed. 0.1 to 0.3 ml of the supernatant were employed for each assay. Duplicate samples of the same liver generally agreed within 10%. Values were compared with those obtained with a highly purified acetyl CoA standard obtained from Dr. Earl Stadtman, who also kindly supplied the crude phosphotransacetylase. Results are recorded as μM CoA per g liver (1 μM = 317 Lipmann units). CoA contents determined by the two methods agreed within 10% and showed no consistent trends in their differences. Most of the results reported here were determined by the Kaplan procedure, involving the acetylation of sulfanilamide.

Results. Livers of the group fed the basal diet (Table I) were classified according to the

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* *Torula* feed yeast 30, sucrose 59, vit. E-free lard 5, salts 5, and vitamin mixture (in lactose) 1; vit. A and D were given orally once weekly(1).

† The vitamin mixture used throughout these studies differs slightly from that previously reported (1). Its composition is: Lactose 88.68 g, thiamine HCl 40 mg, riboflavin 25 mg, pyridoxine HCl 20 mg, D-calcium pantothenate 200 mg, choline hydrochloride 10 g, niacin 1 g, menadione (2-methyl-1,4-naphthoquinone) 10 mg, folic acid 20 mg, biotin 10 mg, vit. B_{12} 1 mg.

TABLE II. Relationship between Metabolic Lesion and CoA Content of Liver.

Supplement added to basal diet			Rats		Liver*	
Kind	Amount, %	Period supplement fed, wk	No.	Final body wt, g	Decline in O_2 uptake in 2 hr, %	CoA content, μM/g
None	—	—	18	80	54	.23 ± .04†
α-tocopherol acetate	50 mg	2	12	86	7	.20 ± .03
		3	10	93	3	.19 ± .03
Factor 3:						
Brewer's yeast autolysate	4 (64 u)‡	3	4	84	13	.25 ± .02
Crude tissue powder	1 (59 u)	3	10	90	22	.19 ± .04
Tissue extract	(29 u)	3	4	88	48	.21 ± .04
L-cystine	1	4	13	112	10	.33 ± .06
DL-methionine	1.23	4	6	111	62	.37 ± .02
L-cysteine	1	4	5	121	71	.38 ± .04
Ca-pantothenate (inj.)	1 mg/day	4 days	9	74	47	.19 ± .04
Stock diet controls	—	40 " old	12	93	0	.45 ± .09

* Normal areas of livers taken for study. † Mean and stand. dev. ‡ u = units of Factor 3(8).

degree of gross pathology. The CoA contents of the livers were markedly depressed when they were grossly necrotic and when the area taken for analysis contained necrotic tissue. However, no differences in CoA contents existed between those livers that had no visible necrosis and appeared to be normal on microscopic examination, and those that had demarcated necrotic areas, if only *normal-appearing* tissue were taken for analysis.

The CoA contents (Table II) of *normal-appearing* areas of livers of rats fed the basal diet are considerably less than those of rats of similar body weight fed the stock diet (0.23 ± 0.04 μM, and 0.45 ± 0.09 μM, respectively). Amounts of α-tocopherol that prevented respiratory decline, as well as liver necrosis, failed to increase the CoA content of the liver. Supplements of Factor 3, which prevented the development of pathological changes during the 3-week repletion period, were also without any effect. Hence, there is obviously no correlation between the development of liver necrosis and the CoA contents of the liver. It is also evident from Table II, that there is no relationship between the CoA level and the defect in oxygen consumption in these groups.

Of the 3 factors protecting against dietary liver necrosis, namely, cystine, vit. E, and Factor 3, only cystine exerts a marked effect on growth. Methionine and cysteine stimulate growth like cystine (Table II) when compared at equivalent levels, but they afford only a fraction of the protective action against liver necrosis obtained by cystine(11). This makes it possible to compare liver CoA values with the growth effect and the protective effect against liver necrosis. Cystine at the 1% level almost completely prevented the occurrence of necrosis and also of respiratory decline. It elevated the CoA value to .33 μM. Methionine and cysteine, which fail to protect against the histologic and metabolic lesions, promoted growth and raised the CoA level of the liver to .37 and .38 μM. This suggests that the liver CoA level is related to the nutritional value of the diet and its capacity to support growth; this, under these conditions, depends on the availability of S-amino acids. The effect is independent of the protective action against liver necrosis. It remains to be clarified whether it is due to the increased intake of S-amino acids *per se,* or that of other dietary constituents.

To show that CoA levels were not limited by the availability of pantothenate, 1 mg of calcium pantothenate was injected intraperitoneally each day for 4 days to rats fed the basal diet. The latter contained more than ample amounts of pantothenic acid (approximately 3.5 mg/100 g diet). The injections of calcium pantothenate had no effect on body weight, the severity of the respiratory decline in liver slices, or on liver CoA contents.

Summary. Rats develop a metabolic lesion

of the liver (respiratory decline in the Warburg) and subsequent dietary necrotic liver degeneration when fed a vit. E-free ration containing 30% *Torula* yeast as the sole source of protein. Depressed liver CoA values are observed in such animals. It is shown that CoA is not elevated by feeding of protective amounts of vit. E or Factor 3. Cystine, methionine, and cysteine, on the other hand, which promote growth under these conditions, raise the liver CoA values to near-normal levels. This action is not correlated to the varying degree of protection against liver necrosis and against the prenecrotic respiratory decline afforded by the individual S-amino acids. Hence, there is no apparent etiological relationship between concentration of CoA in the liver and the development of dietary necrotic liver degeneration.

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Neutralization of Teratogenic and Lethal Effects of Influenza-A Virus in Chick Embryos.*† (21864)

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It has long been known that the course of embryonic development in many species can be altered by the application of various physical and chemical agents, but it has been recognized only recently that certain infectious agents may also produce specific developmental anomalies when introduced into the embryo at a critical time(1-4). Gregg(1) reported that rubella virus infection of women during the first trimester of pregnancy led to the production of cataracts, heart defects, microcephaly, and deaf-mutism in many of their offspring. Hamburger and Habel(2) found that infection of the 48-hour chick embryo with influenza-A virus was followed by microcephaly and micrencephaly, abnormal axial flexion, impairment of amniotic growth, and death within 3 days. This paper shows that these teratogenic and lethal effects induced in the chick embryo by influenza-A virus can be neutralized completely *in vitro*, and to a lesser extent *in ovo*, by specific antiserum.

Materials and methods. Inoculation of embryos. Embryonated hen's eggs incubated for 48 hours were candled to determine the position of the blastoderm, and the air sac was collapsed by gentle suction. An opening was cut through the shell and under 12X magnification test and control solutions were injected through the vitelline membrane immediately covering the embryo, by means of a 30 gauge needle and 0.25 cc syringe combined with a specially designed dispensing apparatus which permitted the injection of 0.03 ml standard

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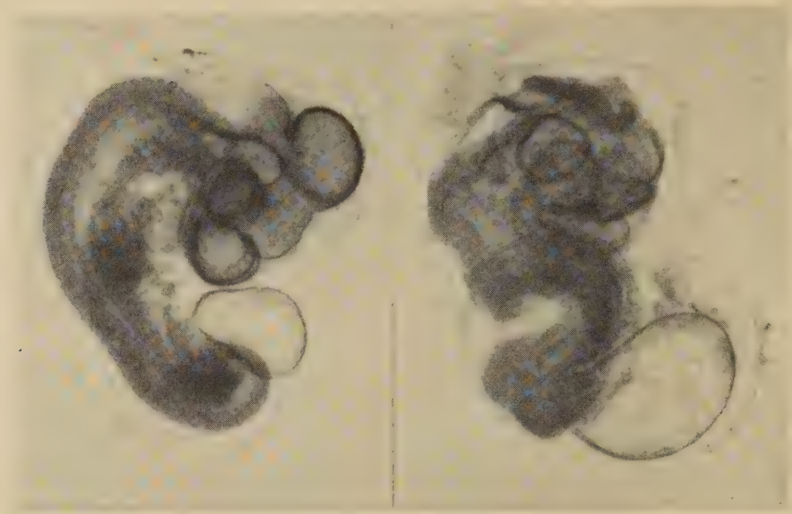

FIG. 1. Chick embryos after 4 days incubation. Control embryo at left shows normal axial curvature and brain development. Infected embryo at right, inoculated 2 days previously with influenza virus, shows characteristic marked axial flexion and micrencephaly.

volume used throughout this study. Following inoculation, the opening in the shell was sealed with scotch tape, and routine precautions against contamination were observed.

Observations of embryos. Daily examinations of the embryos were made by temporarily removing the scotch tape "window." The appearance of the body flexion and/or micrencephaly characteristic of influenza infection, shown in Fig. 1, was recorded as an induced anomaly.

Virus. Infected allantoic fluid which had an E.I.D.$_{50}$ titer of $10^{-6.2}$ served as the source of influenza-A (PR8 strain) virus. The test inocula for chick embryos were prepared by diluting the fluid in Difco Brain Heart Infusion (B.H.I.) medium, and the antigen for immunization of rabbits by diluting it to 10^{-1} with 0.85% NaCl. In both cases the virus suspension was treated with antibiotics by adding 500 units of penicillin and 100 γ of streptomycin per ml of diluent.

Virus recovery. To recover virus from infected tissues, the embryo was minced, ground and suspended in B.H.I. medium 2-3 days after injection of the virus. The suspension, representing approximately a 10^{-2} concentration of embryonic tissue, was inoculated by the allantoic route into a 10-day-old embryonated egg. A positive hemagglutination reaction(5) served to indicate the presence of virus.

Antiserum. A series of intranasal, intravenous, and intraperitoneal injections of virus, administered over a period of 6 weeks, was employed in the preparation of influenza-immune rabbit serum. This antiserum had a hemagglutinin inhibition titer of 1/1600.

Neutralization in vitro. To demonstrate the ability of immune serum to neutralize the effects of the virus *in vitro,* antiserum was incubated in admixture with an equal volume of virus at room temperature for 20 minutes. Prior to mixing the serum had been successively inactivated by exposure to 56°C for 30 minutes, diluted with sterile 0.85% NaCl solution, and treated with antibiotics.

Neutralization in ovo. Undiluted antiserum was administered one hour before or 1, 2, 5, or 8 hours after the injection of the standard virus inoculum.

Controls. Suitable preparations of normal rabbit serum, normal allantoic fluid, or B.H.I. medium, with or without virus, provided control materials.

Results. The virus titration data presented in Table I show that concentrations of virus as low as 10^{-6} were capable of producing anomalies and death. Embryos which received noninfected allantoic fluid developed normally. In most subsequent experiments a

TABLE I. Titration of Influenza-A Virus in 48-Hour Chick Embryos.

Inoculum group	No. of embryos	Effect* observed	Days following inoculation 1	2	3	4	Final incidence, %
10^{-4} Virus	9	(A)	0	9	9	9	100
		(D)	0	3	9	9	100
10^{-5}	9	(A)	0	4	8	9	100
		(D)	0	2	6	9	100
10^{-6}	7	(A)	0	1	2	2	29
		(D)	0	0	1	2	29
10^{-7}	7	(A)	0	0	0	0	0
		(D)	0	0	0	0	0
10^{-8}	7	(A)	0	0	0	0	0
		(D)	0	0	0	0	0
10^{-4} Normal allantoic fluid	8	(A)	0	0	0	0	0
		(D)	0	0	0	0	0

* (A) designates No. of anomalous embryos in each inoculum group while (D) designates No. dead within that group.

10^{-4} concentration of virus was employed as the standard test inoculum because it produced teratogenic and lethal effects uniformly. Virus was recovered from all tissue suspensions which were prepared from dead embryos with typical anomalies. Moreover, concentration of embryonic tissue as low as 10^{-8} was capable of infecting 10-day-old eggs when assayed by the hemagglutination technic. Thus, not only is virus recoverable, but evidently marked propagation occurs during the period in which the pathogenic effects are produced in the embryo.

The data in Table II show that a 1/10 dilution of antiserum was capable of affording complete protection by neutralization of the virus (10^{-2} concentration) *in vitro*. This result did not occur when either normal rabbit serum or B.H.I. medium was substituted for immune serum. Table III shows that dilutions of antiserum as high as 1/800 (before mixture with virus) completely protected all embryos against both the teratogenic and lethal effects of the virus. On the other hand, the employment of either immune serum diluted 1/6400 or higher, undiluted normal rabbit serum, or B.H.I. medium failed to neutralize the virus.

The results obtained when antiserum was administered *in ovo* shortly before or after virus injections are presented in Fig. 2.

When the incidences of anomalies and of deaths are plotted against days after inoculation, it can be seen that the antiserum, although effective, was not capable of preventing the pathogenic effects of the virus in all cases. The optimum protection was obtained when antiserum was administered *before* virus inoculation. The protective effect was less marked when the immune serum was given 1, 2 or 5 hours after virus injection, and it practically disappeared when serum therapy was delayed until 8 hours after virus injection. Control groups exhibited a 100% incidence

TABLE II. Neutralization *in vitro*: Effect of Antiserum.

Inoculum group	No. of embryos	Effect* observed	Days following inoculation 1	2	3	4	Final incidence, %
Virus (10^{-2}) + antiserum (1/10)	22	(A)	0	0	0	0	0
		(D)	0	0	0	0	0
+ Normal serum (1/10)	12	(A)	11	12	12	12	100
		(D)	1	12	12	12	100
+ B.H.I. medium	12	(A)	10	12	12	12	100
		(D)	1	11	12	12	100

* (A) designates No. of anomalous embryos in each inoculum group while (D) designates No. dead within that group.

TABLE III. Neutralization *in vitro*: Titration of Antiserum.

Inoculum group	No. of embryos	Effect* observed	Days following inoculation 1	2	3	4	Final incidence, %
V + A,† undiluted	22	(A)	0	0	0	0	0
		(D)	0	0	0	0	0
1/200	9	(A)	0	0	0	0	0
		(D)	0	0	0	0	0
1/400	8	(A)	0	0	0	0	0
		(D)	0	0	0	0	0
1/800	10	(A)	0	0	0	0	0
		(D)	0	0	0	0	0
1/1600	17	(A)	0	0	0	0	0
		(D)	0	0	1	1	6
1/3200	10	(A)	2	6	6	7	70
		(D)	0	5	6	8	80
1/6400	10	(A)	2	10	10	10	100
		(D)	0	6	10	10	100
1/12,800	10	(A)	2	10	10	10	100
		(D)	0	9	10	10	100
1/25,600	10	(A)	8	10	10	10	100
		(D)	0	8	10	10	100
N.S., undiluted	19	(A)	3	19	19	19	100
		(D)	0	12	19	19	100
B.H.I. medium	24	(A)	13	24	24	24	100
		(D)	0	19	24	24	100

* (A) designates No. of anomalous embryos in each inoculum group while (D) designates No. dead within that group.

† V = Virus (2×10^{-4}); A = Antiserum; N.S. = Normal serum.

of anomalies and deaths. Thus, immune serum can be employed to prevent the detrimental effects of influenza-A virus *in ovo*. However, this protective capacity diminishes with an increased time lapse between injection of the virus and administration of the antiserum.

Discussion. Observations on the development of the early chick embryo infected with influenza-A virus have been made, and the teratogenic and lethal effects of the virus have been successfully neutralized by specific antiserum.

Within 24 hours after injection of influenza-A virus most embryos exhibited an accentuation of the normally gentle curve of the body axis in the thoracic region, frequently accompanied by retardation of the amnion. Rarely were the beginnings of microcephaly present at this time, and deaths had never occurred. By 48 hours after injection, extreme flexion of the body axis was present in more than 97% of the embryos. The amnion usually had failed to close at this time, leaving an aperture of varying size. If closure did occur, the amnion became quite turgid, apparently due to high internal pressure which also severely flattened the posterior half of the affected embryo. More than 95% of the embryos exhibited anomalies of the head region. The mesencephalon and telencephalic hemispheres were typically almost completely collapsed. The eyes were frequently flattened and misshapen, and the auditory vesicles sometimes appeared compressed. About 70% of the infected embryos were dead 2 days after inoculation, while the remainder died during the following 24 hours.

In most instances body flexion and micrencephaly developed before the lethal effect of the virus was manifested. However, on occasion only the axial defect was observed before the embryo succumbed to the infection; rarely did embryos die with micrencephaly in the absence of flexion. Although amniotic defects were present in a high percentage of cases, its retardation was not used as an indicator of infection because of difficulties in observation.

Since the effects of the virus were invari-

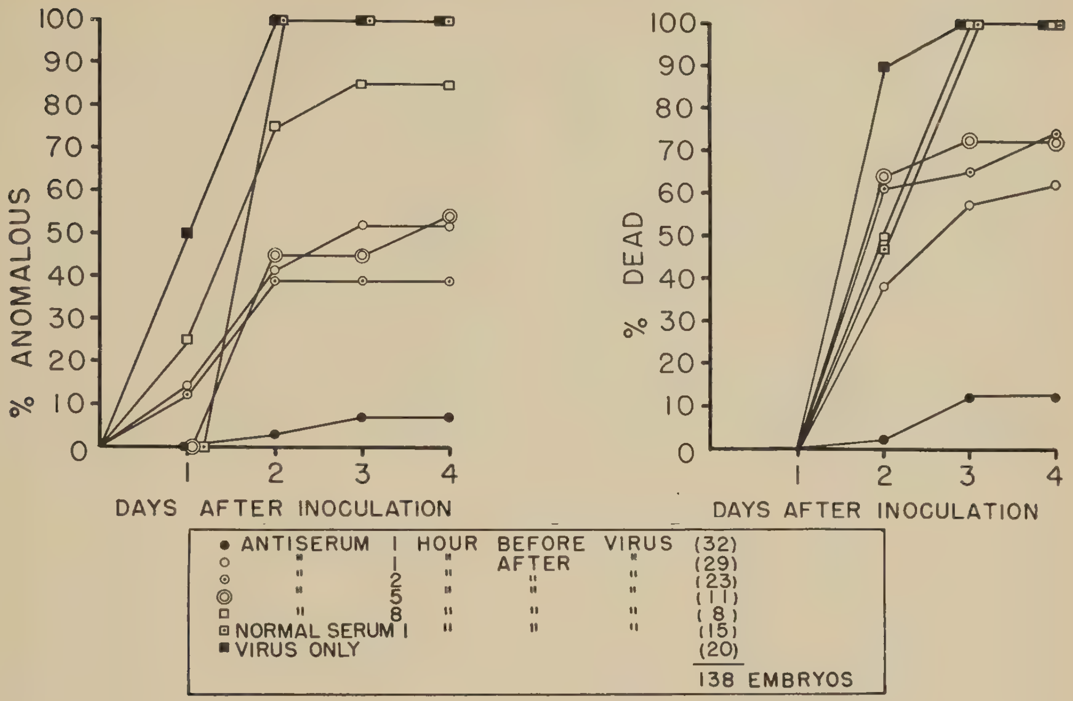

FIG. 2. Neutralization *in ovo* of teratogenic and lethal effects of influenza-A virus.

ably observed within 4 days following injection, anomalies and deaths occurring by the fourth day were presented as the final results in the tabulation of each experiment. It should be mentioned, however, that some deaths occurred 1 to 3 weeks after the start of the experiment among embryos which were apparently developing normally. Anomalies developed in less than 3% of the infected embryos which did not fit the syndrome usually observed. These defects, which were probably the same as the non-viral malformations described by Hamburger and Habel(2), were considered to be the result of operational injury during inoculation, and therefore were not included in the data.

The teratogenic effects of influenza infection may be due to a direct attack by the virus, or its toxin, on those regions of the embryo in which anomalous development is observed. On the other hand, the axial and cephalic defects may be sequelae resulting from retardation of amnion growth by the virus during critical morphogenetic stages. The confinement of a growing embryo within an abnormally small amniotic sheath could lead, through physical compression, to the occurrence of extreme flexion in regions where slight curvature is normally present. The collapse of the brain vesicles and the flattening of the eyes might also be explained on this basis.

Neutralization of the pathogenic effects of influenza-A virus by specific antiserum substantiates the relationship between this virus and the teratogenic and lethal effects observed in the chick embryo. Although serological neutralization of the virus *in vitro* completely prevented the induction of anomalies and death, attempts to neutralize the effects of the virus *in ovo* proved less successful. In accordance with general knowledge concerning the efficacy of serotherapy, maximum protection *in ovo* was obtained when immune serum was given before virus infection, and the protective effect diminished with an increased time lapse between injection of the virus and administration of the antiserum.

Several embryos which received immune

serum treatment *in ovo* died without evidence of anomalous development, despite the fact that virus could be recovered from their tissues. A possible explanation for this phenomenon lies in the knowledge that the chick embryo is susceptible to lethal infection with this virus for at least the first 2 weeks of development, while the period of susceptibility to the teratogenic effects of the virus is restricted to about the first 48 hours of embryonic development.

Conclusions. Specific immune serum will protect the 48-hour chick embryo from the teratogenic and lethal effects produced by infection with influenza-A virus. Inasmuch as antiserum can neutralize these effects completely *in vitro,* and to a lesser extent *in ovo,* this study provides serological evidence to implicate the virus as the cause of the syndrome described.

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Capillary Stress Response and Species.* (21865)

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(Introduced by Charles M. Wilhelmj.)

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Capillary stress response, *i.e.* the changes in capillary resistance occurring upon stress were first observed in the albino rat(1,2). If fully developed, the response consists of two distinct parts, the immediate and late responses. The *immediate response* begins promptly and lasts for 2-4 hours; it is characterized by a sudden rise of capillary resistance with gradual return to former values. The *late response* begins, as a rule, 4-6 hours following stress but is of long (several days or even weeks) duration. It consists of 4 phases: increase, precipitous decrease, period of low resistance, gradual restoration to normal. Whereas the majority of rats responded to stress in the described manner (usual response), occasional animals were found to show only fragments of this "full blown" pattern or no change at all (exceptional response). The human capillary stress response was studied on patients subjected to surgical intervention(3) and on healthy volunteers stressed with ultraviolet light(2,4). Both immediate and late responses were found to show 3 distinct patterns: capillary resistance either increased or decreased or did not change. All 3 patterns were frequent enough to be considered "usual," whereas a biphasic response, reminiscent of the typical late response in the rat, was only exceptionally encountered.

In view of our recent knowledge of the relationship between capillary resistance and certain endocrine functions and of the eventuality that capillary stress response ultimately may reflect changes in the endocrine functions elicited by stress(5,6), it was decided to extend these studies to 3 species of the most commonly used laboratory animals, the dog, rabbit and guinea pig and then to make a comparative analysis of all 5 species.

Method and procedure. The immediate capillary stress response was studied on 23 mature guinea pigs (12 males, 11 females) weighing 400-700 g, on 9 rabbits (5 males, 4 females) of an average weight of 2700 g, and 8 dogs (2 males, 6 females) with a weight ranging from 9 to 11 kg. Forty-one individ-

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ual experiments were made. The study of the late response was performed on 28 guinea pigs (15 males, 13 females), 12 rabbits (6 males, 6 females) and on 13 dogs (5 males, 8 females), with similar weight range, in 57 individual experiments. The following forms of stress were applied. (1) Ether anesthesia for a period varying from 10 to 60 minutes, in all three species. (2) Cold stress in guinea pigs and rabbits (–10°C for 1 hr). (3) Forced muscular work (swimming, guinea pigs, for 1 hr at 37°C, running in the treadmill, dogs,[†] for 30-60 minutes). (4) Anoxia, guinea pigs (exposure to gradually decreasing atmospheric pressure for 30 minutes the deepest point being between 410-260 mm Hg). (5) Ultraviolet light in all 3 species (5-7 minutes at a distance of one foot upon a shaved area with careful protection of the site of testing). (6) Surgical trauma in dogs (unilateral adrenalectomy). (7) Fasting for 14-21 days in the rabbit, for 4-5 days in the guinea pig, for 30 days in the dog (water *ad lib.*, realimentation with the prefasting feed). (8) Emotional stress. All animals were carefully trained prior to experiments so that the handling and the restraint necessary for testing capillary resistance did not engender any emotional strain. The guinea pigs and rabbits assigned for the study of the effect of emotional stress were excluded from this preliminary training. Intensive emotional tension was created in these guinea pigs by tieing them down to a board during the first testing, and in the rabbits by placing them in the treadmill for ten minutes, a procedure which upset them beyond measure. (9) In one dog (weight 10 kg) the effect of sodium phenobarbital anesthesia was studied (625 mg by intravenous infusion during 60 minutes). Capillary resistance was tested by means of the negative pressure method, the details of which were described in previous papers(5,7). The least degree of suction causing one or more petechiae was considered the value of capillary resistance. Dogs and guinea pigs were tested on the abdominal skin, rabbits on the back in the area of the lumbosacral muscles. The hairs were carefully clipped and liquid petrolatum was used for sealing the suction cup and better visualization of petechiae. A control base line of capillary resistance was established prior to the stress and measurements were repeated immediately following it, then 30 minutes, one, two and four hours later (immediate response) and, thereafter, daily for one week (late response). Capillary resistance was also observed *during* the stress when ether or barbiturate anesthesia was administered.

Results. Dogs. The control base line of the dogs studied varied between 15 and 30 cm Hg (mean 20 cm). The *immediate response* was found to be represented by two variants which seemed to be independent of the form of stress applied (ether or phenobarbital anesthesia, running in the treadmill, ultraviolet irradiation). Capillary resistance either increased (7 experiments) or did not change (2 experiments). The increase amounted to 9-21 cm Hg the greatest being following ultraviolet irradiation and the smallest during barbiturate anesthesia. The elevation lasted for between 30 minutes and 4 hours. The *late response* consisted, as a rule, of an elevation of the capillary resistance by 6-21 cm following surgery and treadmill experiment, and by 30-40 cm during fasting. One dog of 13 was encountered with no change in capillary resistance during the stress of fast and one with a decrease following a treadmill experiment. The duration of the elevated capillary resistance varied from 2 to 18 days after surgery. In the fasting animal it remained high during the entire period of fast and returned to the base line after realimentation.[‡] In one dog this return was preceded by a short period of low capillary resistance below the base line thus the entire reaction was similar to the biphasic response of the rat. Fig. 1 shows one experiment with typical immediate and late responses of a kennel reared dog in the treadmill experiment.

[†] Dogs were born and reared in our kennel and protected from any unusual stress for one year prior to experiment. For an ordinary kennel dog, owing to its previous experience, an occasional running in the treadmill would not constitute a stress.

[‡] This was the finding whenever dogs were realimented with balanced kennel diet and the situation may be entirely different with variation of the realimentation diet(8,11).

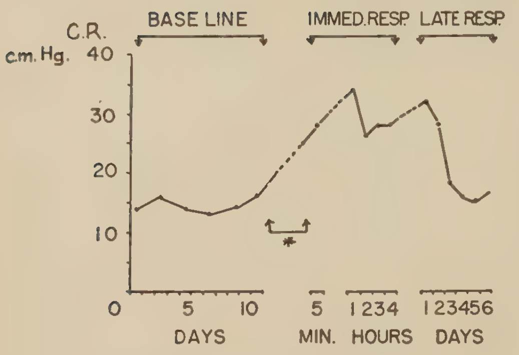

FIG. 1. Immediate and late responses to muscular exercise of a kennel-reared dog (♀ 12 kg) protected from any unusual stress for one year prior to this experiment. Note marked increase in capillary resistance immediately following muscular exercise, and high level persisting for 2 days.

* In treadmill for 1 hr.

Guinea Pigs. The control base line of 28 guinea pigs varied between 17 and 59 cm Hg (mean 33 cm.). Three patterns of *immediate response* were observed: increase of capillary resistance by 6-46 cm in 17 experiments representing all forms of stress applied (ether, cold, swim, ultraviolet irradiation, anoxia), decrease of capillary resistance by 35 cm in one experiment (ultraviolet irradiation), and biphasic response in 4 experiments (2 swim, one anoxia, one ultraviolet irradiation). In 3 experiments the biphasic response consisted of an increase followed by a decrease below the base line, in one experiment the sequence was reversed. The *late response* revealed also 3 patterns: increase, decrease, biphasic response. The predominant pattern was the biphasic one (19 experiments), decrease was observed in 9, increase in 3 experiments. No correlation was found between these patterns and the forms of stress applied except for the 4 cold experiments in which capillary resistance invariably decreased. Remarkable was the biphasic response to fasting and realimentation: capillary resistance increased during the first days of fasting then it decreased below the base line and remained there for 1-3 days after realimentation. Fig. 2 shows a typical cold experiment with both immediate and late responses.

Rabbits. The base line of 12 rabbits was found to range between 40 and 60 cm Hg (mean 55 cm). In 9 experiments in which the *immediate response* was studied to ether, ultraviolet irradiation, cold and emotional stress the patterns of decrease, increase, and biphasic response were encountered. The decrease varied between 10 and 48 cm, the increase between 9 and 23 cm Hg. In the 11 experiments in which the *late response* to ether, fasting, ultraviolet irradiation, cold and emotional stress was observed, the follow-

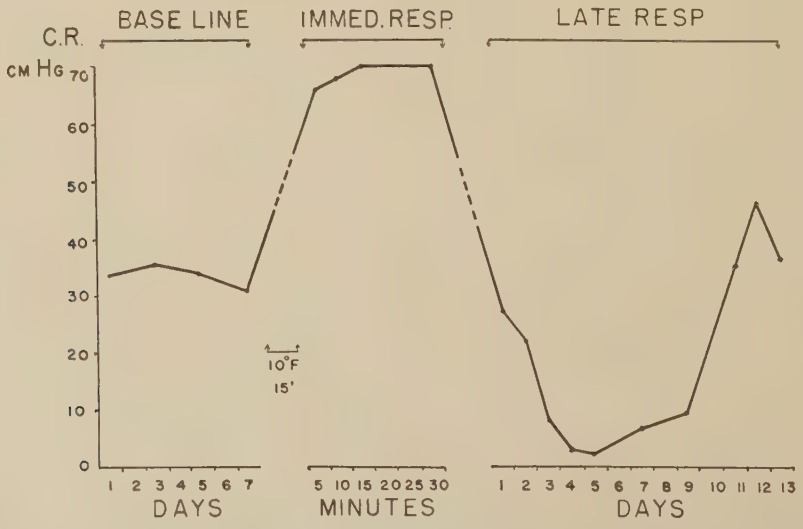

FIG. 2. Immediate and late responses of a guinea pig (♂ 350 g) following exposure to temperature of 10°F for 15 min. Pattern of increase in immediate response and that of decrease in late response are striking.

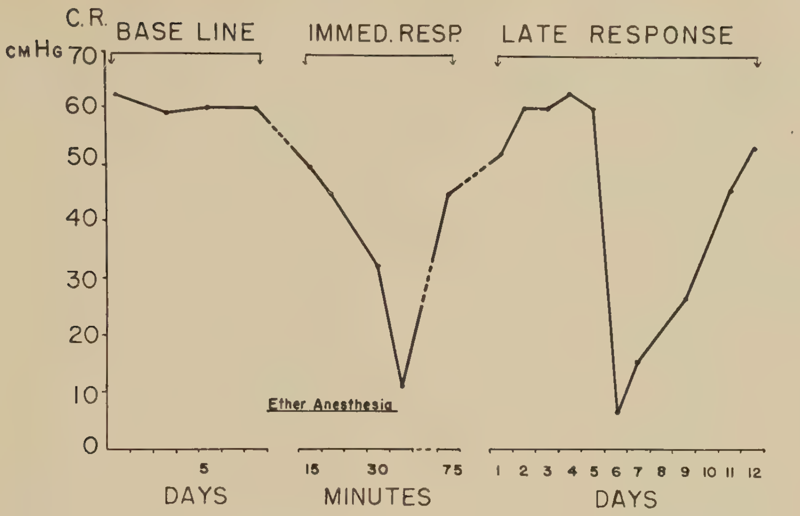

FIG. 3. Immediate and late responses of a rabbit (♂ 2600 g) to ether anesthesia for 30 min. Precipitous decrease in capillary resistance in both parts of stress response. The two parts are well distinguished. Note decrease which sets in *during* stress.

ing patterns were encountered: increase (3), decrease (2), biphasic response (6), no response (1). Fig. 3 shows typical patterns of immediate and late responses to ether anesthesia.

Discussion. In previous studies of the capillary stress response, the rat was first found to respond in a rather uniform manner to various forms of stress. The immediate response consisted of an increase of capillary resistance and the late response was characterized by a biphasic pattern. Subsequently, when these studies were extended to the human subject three patterns were found rather commonly in both, immediate and late responses. This finding seemed first to be controversial to that obtained in the rat but became clarified with increasing experience. Occasional rats were found to respond in a manner formerly thought to be typical of the human and, incidentally, human individuals were encountered showing a response rather reminiscent of the typical biphasic rat pattern. The observations on three further species, described in this paper, intend to complement this picture, enabling us to evaluate capillary stress response on a more uniform basis. The synoptic table may help in obtaining an over-all view. The data in this table referring to humans and the rat are based on 144 and 313 experiments respectively, as reported in previous papers(1-5, 7,8).

At first glance the patterns of capillary stress response seem to be distributed among the 5 species studied in a rather bizarre, haphazard manner, yet these patterns reveal a certain orderliness. They show that any species is capable of producing all variants in the capillary response even though there may be patterns of preference. The essential difference between species is, therefore, that of the incidence in which these patterns occur. One pattern may be "usual" in one species and exceptional in the other and vice versa. This generalization is the least applicable to the immediate response of rats and dogs in which no decrease or biphasic reaction were, so far, encountered. This, however, may be due to the fact that for the demonstration of exceptional patterns a larger series is required.

No relationship seems to exist between the various forms of stress and patterns of response, although certain patterns may be found in a higher incidence following certain types of stress; for example, the capillary resistance of guinea pigs decreased following cold effect more frequently than after any other forms of stress, and an increase in the immediate response of the rabbit was observed, so far, only as a result of ultraviolet irradiation or cold. This, nevertheless, does

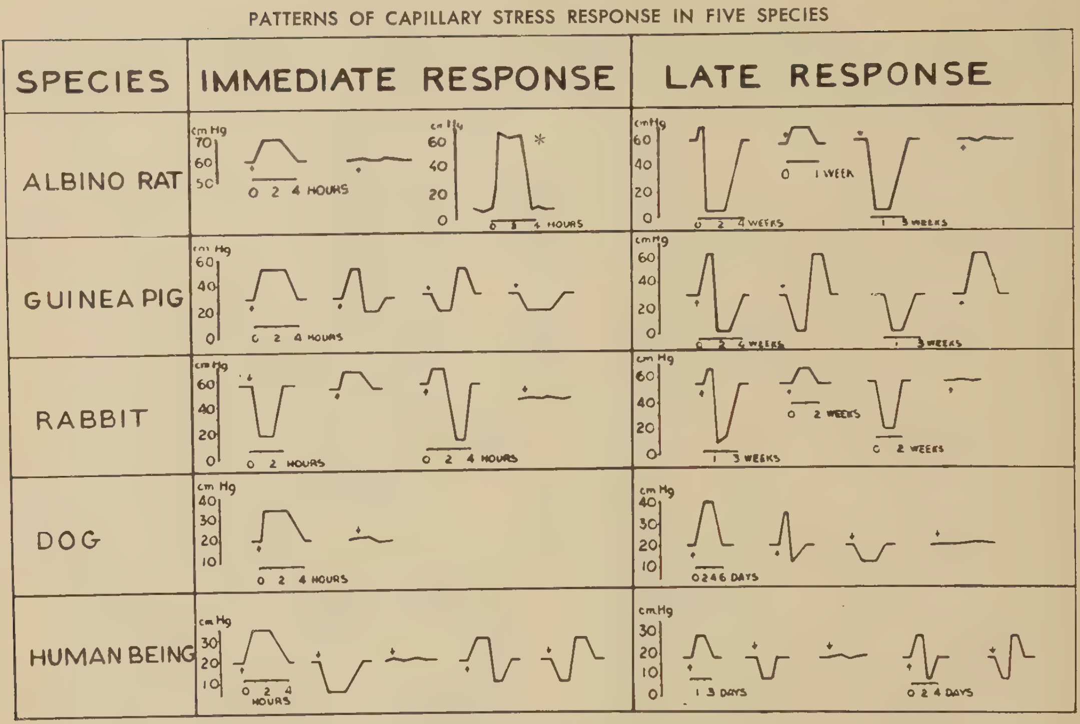

FIG. 4. Patterns are presented in order of their decreasing frequency of occurrence. In the rat the usual patterns of immediate response are shown by the first 2 curves only. Third curve (*) starts with an artificially reduced capillary resistance (adrenalectomy, pretreatment with foreign protein).

not alter the over-all picture which seems to suggest that the capillary stress response in the five species studied is not likely to be basically different.

As to the underlying mechanism of this phenomenon, there is ample proof for ACTH and cortisone respectively and indirect evidence for STH playing an important role in the late response(6,9). Recent studies, in process of publication, suggest that a similar set of 2 capillary active substances, histamine and vasopressin, are at work in the immediate response(10). ACTH by liberation of cortisone is responsible for any increase in capillary resistance occurring *beyond six hours* following stress whereas STH has an opposite effect. For any decrease of capillary resistance which follows stress *promptly*, histamine seems to be accountable. Vasopressin antagonizes this effect of histamine. It is assumed, for the time being as a working hypothesis, that the different patterns of capillary stress response are the result of a balance mechanism of these two sets of capillary active substances and that this balance is different in various species. The possible involvement of the nervous system in this balance mechanism is the subject of a study in progress.

There can be no doubt that knowledge of the capillary stress response is essential in any experimental or clinical study in which testing of capillary resistance is involved. Ignorance of this phenomenon and failure to avoid or, at least, to recognize it will necessarily engender errors in the evaluation and interpretation of the results.

This comparative study of stress response enables us to pass a judgment on the relative value of these 5 species as experimental subjects for the study of capillary resistance in general. In our experience, the *human being* and the *dog* are the most suitable species. Their control base lines, as a rule, do not show too wide fluctuations and the level of capillary resistance is such that both increasing and decreasing effects may well be studied. The formerly described phenomenon of spasm of the precapillary arterioles in vasolabile individuals(7) is not particularly disturbing, provided that it is recognized. The well-trained *rat* is an excellent subject but because of its inherently high capillary resistance level any further rise cannot be always reliably demonstrated. It is very suitable for the study of any factors which are likely to decrease capillary resistance. The rat lends itself excellently to endocrine studies of capillary resistance, especially after adrenalectomy or hypophysectomy. Although capillary spasm not infrequently occurs, in well-trained animals it is no major obstacle. *Guinea pigs* may sometimes be preferred because of their more medium basic capillary resistance level, yet they have the disadvantage of being excessively influenced by emotional or environmental factors, as shown by the frequent occurrence of capillary spasm and by the fact that a training similar to that applied in a rat is often not enough to prevent emotional capillary stress response elicited by and interfering with the experiment itself. Very tame individuals with a body weight of more than 600 g, however, may prove to be excellent. In our experience, the *rabbit* is the least suitable subject for capillary resistance studies. Besides their great emotional lability, they can hardly be trained to be tested on their abdomen, so the back must be used. When clipping the hairs their skin is easily irritated and often shows small maculopapular blemishes which can only be distinguished from true petechiae by the use of pressure with a glass slide. Great care must also be taken to keep the skin taut lest doming into the suction cup may occur to such an extent that it interferes with circulation and accurate readings.

Summary. 1. Changes in capillary resistance resulting from 8 forms of stress were studied on dogs, guinea pigs and rabbits in order to integrate previous similar studies on the human being and the rat. 2. Immediate and late responses were investigated and essentially the same patterns of response found as previously described. A comparison of the results obtained on all five species studied shows that practically every pattern may occur in each of the 5 species, yet in each species certain patterns predominate. Thus, the species difference resolves itself into a difference in the incidence in which the various patterns occur. 3. Knowledge of the capillary stress response is indispensable in any clinical or experimental study in which capillary resistance is measured. 4. An evaluation of the five species is given as to their suitableness to and relative value in capillary resistance studies.

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Effects of Unilateral and Bilateral Carotid Arterial Occlusions in Monkeys.* (21866)

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Reports concerning the effects of permanent carotid arterial occlusions in primates have been infrequent. During a study concerned with ligations of major cerebral arteries in monkeys, it appeared desirable to determine the clinical and electroencephalographic effects of acute permanent occlusions of the carotid arteries. The following experimental studies were therefore undertaken.

Method. Bilateral carotid artery ligations were performed on 13 *Macaca mulatta* (2.5-5.0 kg) which had previously undergone brain operations for the production and modification of chronic experimental epilepsy following intracranial application of alumina cream by injection or disc(1,2).

In *Group A,* consisting of 5 monkeys, both common carotid arteries were doubly ligated at a low cervical level with linen ties in 1 stage.

In *Group B,* consisting of 4 monkeys, both common, internal and external carotid arteries were ligated in 1 stage. Common carotid arteries were ligated as in Group A and, in addition, internal and external carotid arteries were ligated in the upper neck just above their origin from the common carotid. In *Group C,* consisting of 4 monkeys, carotid ligations were carried out as for Group B, but in 2 stages so that the common, internal and external carotid arteries of one side were ligated 6 weeks (2 monkeys) or 18-21 months (2 monkeys) following previous similar ligation of the opposite sided carotid vessel. Serial *clinical* and *electroencephalographic* observations were made on surviving animals. Postmortem examinations included gross and histologic check of ligated vessel areas.

Results. In Group A, in which both common carotid arteries were ligated in 1 stage, 4 of the 5 animals survived. The fifth monkey died 2 days postoperatively and at postmortem examination showed a moderate sized acute right epidural hemorrhage. No significant changes were noted clinically in the 4 survivors. Electroencephalograms in 3 of this group showed no additional change in serial recordings beginning on first postoperative day compared with recording made day prior to operation. In one monkey accentuation and increase of slow frequencies over the side of previous cerebral operations persisted for 3 weeks. In the monkey which expired, EEG on 1st postoperative day disclosed diffuse moderate increase of slow high amplitude frequencies, more pronounced over the side of the epidural hematoma.

In Group B, in which both common, internal and external carotid arteries were ligated in 1 stage, 3 of the 4 monkeys expired within 48 hours. These animals appeared listless, weak, dyspneic, drowsy or semicomatose following operation, with increasing progression of these signs until death. EEG's on 2 of this group 24 hours postoperatively showed either no significant added change or mild grade of increased diffuse slowing compared to the EEG made on day prior to operation. In the surviving animal, no clinical or electroencephalographic abnormalities were noted.

In Group C, in which the major carotid arteries of both sides were ligated in 2 stages, death occurred 6-7 days postoperatively in the 2 monkeys in which the interval between right and left sided carotid arterial ligations was 6 weeks. Both monkeys became increasingly lethargic, weak, anorexic, and dyspneic following operation. EEG taken 24 hours postoperatively showed no added change in 1 and increased slow wave frequencies of moderate degree over the brain operated side in the other. In the remaining 2 monkeys of this group in which the intervals between the right and left sided carotid ligations were 18

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and 21 months, no clinical, behavioral or electroencephalographic changes were noted compared to the status immediately prior to the operations. No detectable change occurred clinically or in EEG after the initial single sided common, internal and external carotid ligations in any of the 4 monkeys of this group.

The clinical epileptic status of the monkeys did not alter significantly following unilateral or bilateral carotid arterial ligation.

Discussion. These results indicate that abrupt permanent occlusion of both common carotid arteries (Group A) is generally well tolerated by *Macaca mulatta.* In such animals collateral circulation to the brain may take place via retrograde external carotid flow into the internal carotids as well as through the vertebral-basilar system. When the internal and external carotid arteries as well as the common carotids were also ligated abruptly (Group B) the procedure was much less readily tolerated. In such animals collateral circulation probably comes mainly through the vertebral-basilar arterial system. Since only 1 of 4 monkeys survived it might be inferred that in the survivor, the basilar arterial circulation with its contribution to the Circle of Willis was proportionately higher than the others. This view is supported by the findings of Dumke and Schmidt(3) who indicated that the basilar artery contributes a highly variable portion of the total cerebral blood flow; their quantitative measurements of cerebral blood flow in Macaque monkeys showed that the basilar:carotid ratio varies widely (from 26 to 80% when blood flow is measured simultaneously in the basilar and carotid arteries.)

When a prolonged interval of time (18-21 months) elapsed between right and left common, internal and external carotid ligations, no sequellae of consequence were noted in clinical status or EEG. In these animals it is concluded that a sufficient time interval had been allowed for adequate collateral circulation to build up. Such collaterals could occur not only through the vertebral-basilar channels but also through the relatively smaller anastomotic connections between the internal and external carotid arteries, similar to those cited by Weiss(4). In 2 stage ligations it would appear that a period greater than 6 weeks is necessary for establishment of adequate collateral circulation since neither of the 2 animals survived in which the interval between right and left common, internal and external carotid was 6 weeks.

The EEG made on 1st postoperative day did not prove to be a particularly useful indication of the ultimate fate of a monkey after bilateral carotid arterial occlusion. This was especially noteworthy in Groups B and C in which little or no significant EEG alteration was noted on the first post-operative day in animals which died 1 to 6 days later.

Summary. The effects of acute permanent carotid arterial occlusion were studied in a group of 13 healthy *Macaca mulatta* which had sometime previously undergone brain operations for the production of chronic experimental epilepsy. (1) Of 5 monkeys in which both common carotid arteries were ligated simultaneously, 4 survived without significant clinical or EEG change while the 5th expired on the 2nd postoperative day. (2) Of 4 monkeys in which both common, internal and external carotid arteries were ligated simultaneously, 3 expired on 2nd postoperative day while the survivor exhibited no significant clinical or EEG change. (3) In 4 monkeys in which the common, internal and external carotid arteries of one side were ligated, all survived without significant clinical or EEG change: (a) when the opposite common, internal and external carotid arteries were subsequently ligated after 18 to 21 months in 2 of these animals, no significant clinical or EEG change occurred; (b) when the opposite common internal and external carotid arteries were subsequently ligated 6 weeks later in the remaining 2 monkeys, death occurred in 6-7 days.

Conclusions. It is concluded that acute permanent unilateral complete carotid (common, internal and external) or bilateral common carotid occlusions are well tolerated by monkeys. However, bilateral acute permanent complete carotid (common, internal and external) occlusions are only occasionally well tolerated. Bilateral complete carotid occlusions are more likely to be well tolerated if right and left sides are ligated separately with a prolonged interval (over 6 weeks) between the 2-sided occlusions to permit establishment of adequate collateral circulation.

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Tonic Convulsions Induced in Mice by Vaccinia Virus and Their Prevention By Heated Influenza Virus. (21867)

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(Introduced by Bradford N. Craver.)

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During routine investigations of a mouse-adapted strain of vaccinia virus it was observed that mice infected intracerebrally with this virus exhibited severe tonic convulsions when suspended by their tails and twirled vigorously. Since convulsions resulting from vaccinial infection of man and animals have been only irregularly observed(1,2) it seemed desirable to investigate this phenomenon further. The following report will demonstrate that certain strains of vaccinia virus regularly produced tonic convulsions in mice. Evidence is also presented indicating that these convulsions occurred after the virus had multiplied extensively in the brains of the mice. This latter fact differentiates tonic convulsions produced by vaccinia virus from those produced by massive intracerebral doses of egg-adapted influenza viruses, which apparently multiply very little if at all in the mouse brain(3,4). Additional data show that the tonic convulsions induced by vaccinia virus were abolished if the mice received prior intracerebral injections of heat inactivated influenza virus.

Methods. The majority of these studies involved a single frozen pool of the New York Board of Health (NYBH) strain of neurovaccinia virus adapted to mice after numerous mouse brain passages. Although this pool of 20% homogenized, infected mouse brains had been stored at –70°C for 6 years before use, new pools made from this material exhibited equivalent characteristics. For comparative purposes some experiments included the WR (Western Reserve) strain of neurovaccinia virus (American Type Culture Collection) which was also originally derived from the NYBH strain. This pool of the WR strain was prepared from infected mouse brains which had been homogenized in tryptose phosphate broth, diluted to a 10% suspension, sealed in glass ampules and, as with all the other viruses used, stored at –70°C. The mice (18 to 21 g Webster strain) used in these experiments, while under light ether anesthesia, were injected into the right brain hemisphere with 0.03 ml (0.05 ml in one case) of vaccinia virus preparations. Embryonated eggs were infected with vaccinia virus by either depositing 0.05 ml of viral material on the "dropped" chorio-allantoic membranes (CAM) of 10-day-old eggs or by injecting 0.5 ml of the viral preparation into the yolk sac (YS) of 7-day-old eggs as described in a previous report(5). All viral preparations used in the following experiments had been shown to be free of bacterial contamination.

Experimental. Tonic Convulsions. Table I contains typical data illustrating the occurrence of tonic convulsions in mice injected intracerebrally with vaccinia virus. In all experiments involving tonic convulsions as an end point (50% tonic convulsive dose = TCD_{50}) the convulsions were precipitated by suspending the mice by their tails twice daily and twirling vigorously 20 times, a technic

TABLE I. Occurrence of Tonic Convulsions in Mice Injected Intracerebrally with Various Strains of Vaccinia Virus.

Virus strain	Type of preparation	Dilution	Results in mouse No. 1	2	3	4	5	6	7	8	9	10
NYBH, mouse-adapted	20% mouse brain	10^{-3}	D_4	D_4	D_4	C_4	C_4	C_4	C_4	C_4	C_4	C_5
WR mouse neurotropic	10% mouse brain	10^{-3}	C_5	C_5	C_5	C_5	C_5	C_6	C_6	D_6	S	S
NYBH, egg-adapted	10% CAM* suspension	10^{-1}	D_4	C_4	C_4	C_5	—	—	—	—	—	—

D = spontaneous death; C = induced tonic convulsion; S = mouse survived 11 days post infection.

Subscript numerals indicate No. of days after infection.

* Pool of chorio-allantoic membranes.

discussed in former reports(3,6). As the data indicate some mice expired during those periods when no observations were made. For the sake of convenience such deaths were included in all calculations of the 50% tonic convulsive dose. It was also observed that with few exceptions the mice died as a result of the induced tonic convulsions. In some experiments in which only the 50% lethal dose (LD_{50}) was determined these mice were not twirled and deaths were permitted to occur spontaneously; it was not established, however, that all such deaths were the result of tonic convulsions. Numerous experiments indicated that either a 10^{-3} or a 10^{-2} dilution of vaccinia virus regularly produced either tonic convulsions or death in all the mice but not within the first 48 hours after infection and rarely during the first 72 hours.

The data in Table II indicate the ability of various virus strains to produce tonic convulsions in mice, and death in both mice and chick embryos. The data for Experiments 4 and 5 indicate that the TCD_{50} and the LD_{50} produced by the mouse-adapted NYBH strain in mice were similar. The data also indicate that chick embryos were at least as sensitive as mice to both the WR and NYBH strains of vaccinia virus and occasionally appeared more sensitive than mice. Although the WR strain of neurovaccinia virus had a reasonably high titer in mice as expected, it will be noted that the NYBH egg-adapted strain also produced tonic convulsions (Table II). To our knowledge, this strain had always been passed in eggs but like strains passed in mice, it induced the same type of tonic convulsions at dilutions similar to those of the mouse-adapted strains.

As mentioned in previous reports(3,4) the egg-adapted strains of influenza virus either did not multiply or multiplied meagerly in the brains of mice but in high concentration regularly produced tonic convulsions. Although it could be assumed that the mouse-adapted vaccinia virus multiplied in mouse brain it still seemed pertinent to substantiate this assumption and relate the viral multiplication to the incidence of tonic convulsions. The data in Table III indicate that the NYBH strain did multiply to a significant degree as evidenced by the LD_{50} of infected mouse brains titrated in both eggs and mice. In these experiments mice were injected intracerebrally with 0.03 ml of a 10^{-3} dilution of infected mouse brain suspension. At the indicated hours groups of 5 mice were sacrificed,

TABLE II. Comparison of the Titers of Various Strains of Vaccinia Virus in Mice and Eggs.

Strain	Mice TCD_{50}*	Mice LD_{50}*	Eggs LD_{50} via CAM†	Eggs LD_{50} via YS†
NYBH mouse-adapted	4.7‡	—	—	—
	4.0‡	—	—	—
	4.9‡	—	—	—
	4.9	5.0	6.5	6.4‡
	4.8	4.3	5.0	—
WR	4.3	3.3	4.0	—
NYBH egg-adapted	3.4	—	—	—

Figures are reciprocal of log of that dilution which produced a 50% end point as calculated by method of Reed and Muench(7). All mice injected intracerebrally with 0.03 ml of each of above pools which were the same preparations as indicated in Table I.

* 50% tonic convulsive dose, including spontaneous deaths. To determine the LD_{50} the mice were left undisturbed. Experiment terminated 10 days post infection.

† For method of egg inoculation see *Methods*. CAM = chorio-allantoic membrane; YS = yolk sac.

‡ Ten mice or eggs were used for each dilution in determining these end points, all others involved groups of from 5 to 7 mice or eggs.

TABLE III. Titrations in Eggs and Mice of Infected Mouse Brains Harvested at Various Periods after Infection with a 10^{-3} Dilution of the NYBH Strain of Neurovaccinia Virus.

Hr after infection*	Exp. 1		Exp. 2	Cumulative % of 20 mice induced to tonic convulsions (includes spontaneous death)
	Mice†	Eggs	Mice‡	
0	0	0	—	0
2	0	2.4	—	0
24	0	2.7	1.6	0
48	4.2	3.6	>4.0	0
72	5.7	4.5	>4.0	5
96	3.8	4.3	>4.0	50
120	—	—	—	82

Figures are reciprocal of log of that dilution producing 50% end-point. Experiments were terminated 10 days post infection.

* Pools of 5 brains harvested at indicated hours.

† Mice inj. intracerebrally with 0.05 ml of each 10-fold dilution of every pool of 5 brains to be titrated; eggs received 0.5 ml via the yolk sac.

‡ These mice received 0.03 ml of each dilution intracerebrally.

their brains removed aseptically and stored at −70°C. Just before titrating, a 10% homogenate of each pool of 5 brains was prepared in tryptose phosphate broth and, after proper 10-fold dilutions had been made, injected into both eggs and mice. Even though vaccinia virus is extremely stable precautions were taken to maintain equally for each pool the interval between the removal of infected brains and their titration. Although the parallel titrations, determined in both mice and eggs (Table III), did not always agree, this does not alter the fact that considerable viral multiplication occurred in the brains of the infected mice. The last column of this table was included to permit comparing the incidence of tonic convulsions in a group of 20 mice with the rise in viral titer in mouse brain. Apparently the majority of tonic convulsions occurred sometime after the virus had achieved its maximum titer.

In several experiments the characteristics of the mouse-adapted NYBH strain of vaccinia virus were compared to those of the WR strain, to determine whether the NYBH strain exhibited those qualities usually ascribed to neurovaccinia viruses. It was found that both strains produced typical vaccinial pox on the chorio-allantoic membrane of eggs and essentially equivalent reactions in mice. As will be discussed below, the NYBH strain induced cross immunity in mice to the WR strain. Preliminary neutralization tests indicated that serum from a rabbit immune to the NYBH strain neutralized the infectivity of the WR strain for both eggs and mice. The NYBH strain also produced typical vaccinial lesions on rabbits infected dermally.

Interference by influenza virus. A recent report(8) showed that inactive influenza virus (PR8) interfered with the multiplication of vaccinia virus on the chorio-allantoic membrane of eggs. This interference was also demonstrated in mice using influenza B (LEE) virus (Table IV). In these experiments the influenza virus was injected as a 10^{-5} dilution in 0.5 ml amounts into the allantoic cavity of 10-day-old eggs. The eggs were then incubated for 48 hours at 37°C, after which they were chilled before the allantoic fluids were harvested. Two portions of this pooled allantoic fluid, showing a 50% infectious dose for eggs at a dilution of $10^{-9.5}$, were heated at 56°C for ½ and 1 hour, respectively, then injected, undiluted, in 0.03 ml quantities into the left brain hemispheres of mice. Twenty-four hours later the opposite brain hemispheres were injected with a 10^{-3} dilution of mouse-adapted vaccinia virus (NYBH). The data show that essentially all mice survived and no tonic convulsions could be induced. The influenza virus apparently protected the mice whether it was heated for ½ or 1 hour. Three weeks later these protected mice were mixed and then divided into 2 equal groups, one group was challenged again with the NYBH strain of vaccinia virus, the other group with the WR strain. The mice were immune to both strains. Normal mice of equivalent size and age, however, succumbed to infections caused by either vaccinial strain. It is of interest to note that heated vaccinia virus (as mouse brain suspension) apparently also produced some degree of interference (Table IV). Heated suspensions of normal mouse brain and heated normal allantoic fluid failed to protect mice.

Wagner has shown(9) that sublethal intracerebral doses of influenza virus or receptor destroying enzyme (RDE) produced by

TABLE IV. The Protective Effect of Heated Influenza (LEE) and Heated Vaccinia (NYBH) Viruses on Tonic Convulsions and Death Produced by Vaccinial Infection in Mice.

Material inj. intracerebrally 24 hr prior to infection	Survivors/Total* Exp. 1	Exp. 2
Untreated controls	0/10	0/7
LEE, heated ½ hr at 56°C†	10/10	8/8
LEE, ” 1 ” ” ”	9/10	—
NYBH, ” ½ ” ” ” ‡	7/10	4/8
Heated 20% normal mouse brain	0/10	—
Heated normal undil. allantoic fluid	1/10	—

Each mouse inj. with 0.03 ml of the NYBH strain (unheated).

* Surviving mice showing no convulsions/total mice, 10 days after infection.

† As undiluted allantoic fluid.

‡ As 20% mouse brain suspension.

Vibrio cholerae injected intracerebrally prior to challenge with neurotoxic quantities of influenza virus protected all mice from tonic convulsions and death. Although RDE was not examined for its protective effect in mice infected with vaccinia virus, Depoux and Isaacs(10) did find that this enzyme had no effect on vaccinial infection in eggs. It has also been reported(6) that the microbial product xerosin protected mice from tonic convulsions and death induced by influenza virus. A number of experiments were undertaken to find if xerosin exhibited a protective effect on mice infected with vaccinia virus and it was found that, in this case, xerosin had no effect on the incidence of tonic convulsions.

Discussion. Convulsions are often present in post-vaccinial encephalitis in humans(1). Apparently convulsions are also a regular and inducible phase of infections in mice with certain strains of vaccinia virus. There was a sharp contrast, however, between reported tonic convulsions produced by influenza virus and those resulting from vaccinial infection in mice in that: 1. vaccinia virus multiplied, 2. xerosin protected against influenza virus but not vaccinia virus, and 3. vaccinia virus produced tonic convulsions in a relatively high dilution, whereas massive doses of influenza virus were required to produce a similar effect in mice. Since the Henles attributed the neurological activity of influenza virus to a toxic effect of the injected viral material and not to its multiplication(3) this may be a factor with the vaccinia virus induced tonic convulsions, even though this virus multiplied. Additional experiments are required to resolve the question of whether cellular destruction caused by vaccinia virus multiplication or a toxicity due to accumulated quantities of virus was responsible for the convulsions and death observed.

The protection of mice by heat inactivated influenza virus from the effects of vaccinial infection cannot be explained by the receptor destroying enzyme of this virus, since this enzyme would have been inactivated at the temperature used. Perhaps, as Depoux and Isaacs suggested(10), the heated influenza virus particle may compete with the vaccinia virus particle for an essential site within the brain cells of the mice.

Summary. The reported experiments have demonstrated that: (1) various strains of vaccinia virus after intracerebral injection regularly produced tonic convulsions in mice; (2) at least one of these strains (NYBH) multiplied extensively in the brains of the mice; (3) mice were protected from the tonic convulsions and death caused by intracerebral injections of vaccinia virus, if they had received a prior injection of either heated influenza virus or heated vaccinia virus.

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Hepatic Nodules Resembling Tumors in Rats after Administration and Withdrawal of Ethionine. (21868)

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This paper is the report of the development of nodules resembling tumors in the liver following the feeding of synthetic diets containing ethionine (alpha-amino-gamma-ethyl-mercaptobutyric acid), a methionine antagonist. These nodules were of two types, in that some closely resembled hepatomas, although more commonly they resembled cholangiomas.

Material and methods. Male albino rats were fed *ad libitum* a synthetic diet consisting of 67% glucose, 18% casein, 11% corn oil (containing 0.001 cc haliver oil) and 4% salt mixture(1). The following amount of crystalline vitamins were added per 100 g of diet: thiamine chloride 400 μg, pyridoxine hydrochloride 400 μg, riboflavin 800 μg, calcium panthothenate 1.5 mg and nicotinic acid 2.5 mg.

The animals were divided into 5 groups. Group 1 (25 animals) received the basic diet for 28 days and served as a control for Group 2 (28 animals) which received the basic diet supplemented with 0.5% ethionine. Groups 3 (14 animals) and 4 (23 animals) were similar to Groups 1 and 2 respectively, except that they were maintained on the diet for 63 days and then autopsied. Group 5 (8 animals) received the ethionine supplement for 28 days and then returned to the basic control diet for 35 days, after which time the animals were killed for study. The latter group was on the experiment for a total of 63 days and can be compared with Groups 2 and 4 that were fed ethionine for 28 and 63 days respectively. Four random sections were taken of the liver and stained with hematoxylin and eosin, Sudan III, Wilder reticulum stain and Mallory's aniline blue-stain.

Results. After 28 days marked changes were seen in the liver and pancreas in the group fed the ethionine supplement (Group 2). These changes were similar to those previously described by others(2-7). However, in spite of the marked regeneration in the

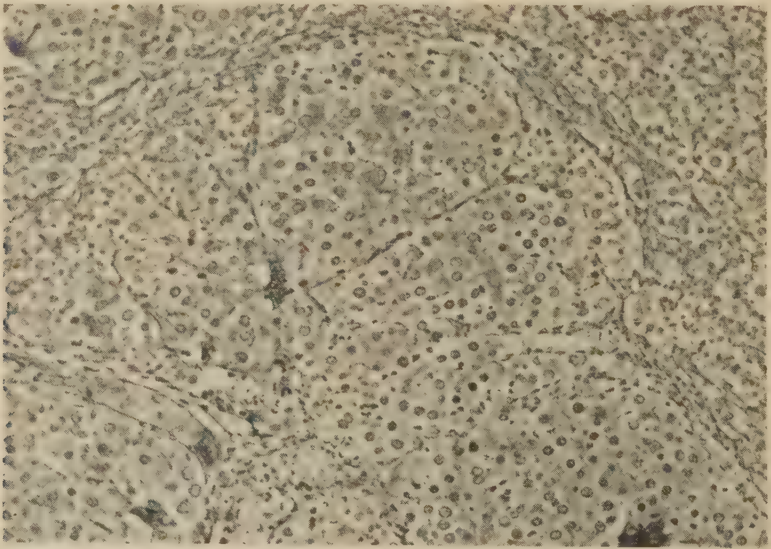

FIG. 1. A large nodule resembling a hepatoma. Nodule has compressed the liver tissue and is well demarcated from adjacent surrounding liver. Group 4 after 63 days of ethionine feeding. Wilder reticulum stain, 100×.

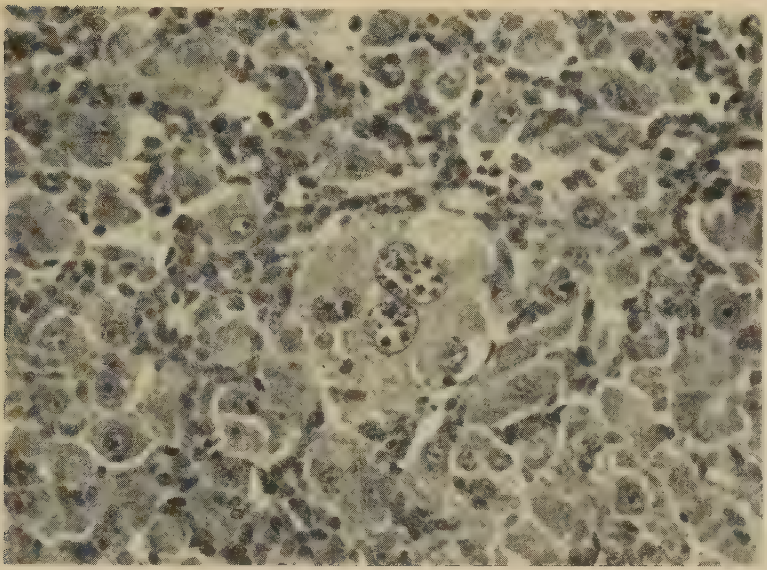

FIG. 2. A small nodule resembling a hepatoma and consisting of only 4 cells on section. Cells of nodule are large, pale and vacuolar. Large vesicular nuclei contain multiple prominent nucleoli and irregular clumps of chromatin. Group 4 after 63 days on ethionine. Hematoxylin and Eosin, 360×.

liver, no nodules were seen in any of the experimental animals in this group. No lesions were found in the 25 control animals on the synthetic diet (Group 1).

After 63 days, nodules varying in size from 83 μ to 1.346 mm were seen in 16 out of 23 rats upon microscopic examination of the liver. In 3 of the 16 rats the nodules resembled hepatomas and consisted of groups of parenchymal cells which closely resembled one another. These nodules compressed and were well demarcated from the adjacent surrounding liver (Fig. 1). This was confirmed by the reticulum stains. The nuclei were large and vesicular and contained single or multiple large, prominent nucleoli while the cytoplasm was abundant, vacuolated and stained a pale pink with eosin. The smallest such nodule consisted only of 4 cells on section (Fig. 2). The adjacent liver cells stained more darkly and the general liver architecture could still be recognized. Proliferation of the bile ducts was striking and diffuse, as was the inflammatory response.

Nodules resembling cholangiomas were observed in 10 animals. These nodules contrasted sharply with the adjacent tissue, although they were somewhat irregular in outline and did not cause any apparent compression. The distorted bizarre bile ducts in the nodules were lined by cells which, in many respects, were quite different from the adjacent diffusely regenerating bile ducts. Within the nodules, whole or portions of bile ducts were lined by large, crowded intensely basophilic cells with large, hyperchromatic nuclei. There was a marked alteration in the nuclear cytoplasmic ratio to such an extent that in some of the cells the cytoplasm was hardly discernible. Some of the ducts contained pink-staining amorphous material and polymorphonuclear leukocytes (Fig. 3). In 3 other animals the liver contained both types of nodules, that is, the hepatoma type and the cholangioma type. The 14 control animals showed no lesions (Group 3).

In the group in which ethionine was withdrawn from the diet after 28 days and the animals killed 35 days later, the livers in 5 of 8 animals contained nodules resembling cholangiomas (Group 5). These were similar to those described in the animals on the diet for 63 days (Group 4). However, the surrounding liver differed in that regeneration was complete and closely resembled the controls (Fig. 4). A moderate amount of fatty

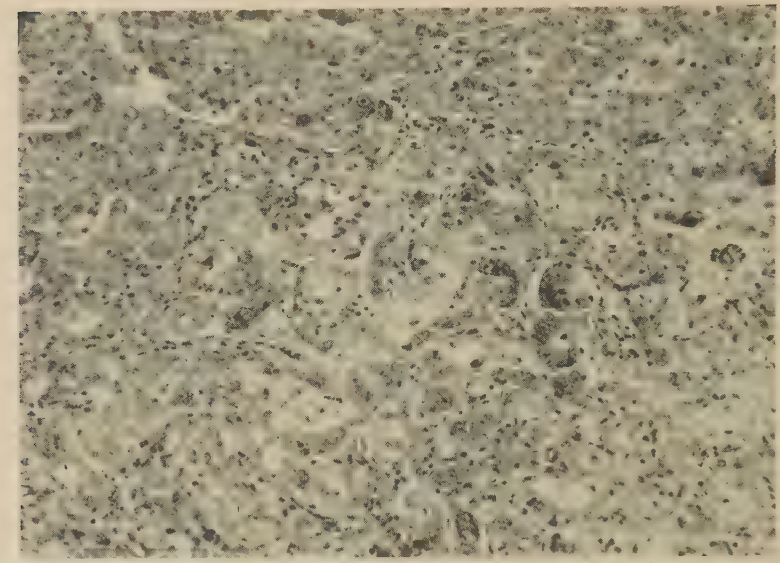

FIG. 3. Nodule resembling cholangioma. Distorted, bizarre bile ducts are lined by large, intensely basophilic cells with large, hyperchromatic nuclei. Group 4 after 63 days on ethionine. Hematoxylin and Eosin, 360×.

metamorphosis was present in one case.

Discussion. Nodules similar to those reported here have been described by Popper, *et al.*(8) after administration of ethionine for 51 to 104 days. This was prevented by methionine. Alvizouri and Warren(2) mention the formation of a cholangioma in one animal in their series. In our experiment no nodules were seen when animals were fed ethionine for 28 days and then killed for histologic study, but after 63 days the nodules were apparent. It is not certain whether these nodules represent areas of atypical regeneration or whether they are the true counterparts of hepatic car-

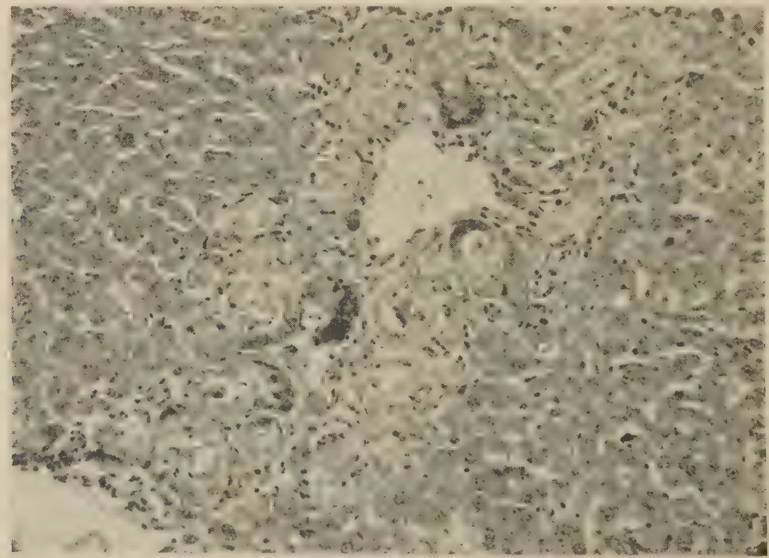

FIG. 4. Nodule resembling cholangioma. Nodule essentially similar to that in Fig. 3. Surrounding liver is almost completely regenerated. Group 5, 63 days after experiment was started and 35 days after ethionine feeding ceased. Hematoxylin and Eosin, 185×.

cinoma as seen in humans. No metastases were found; however, the nodules were small and of short duration.

The most striking thing in this experiment is that the animals did not have to be exposed to ethionine for a long period of time in order to bring about the eventual development of hepatic nodules. The rats fed ethionine for 28 days and then returned to the control diet, free of ethionine, for 35 days, developed nodules by the end of the 63rd day in approximately the same proportion of cases and of the same size as when ethionine was fed for the entire 63-day period. It is presumed that the nodules did not develop before 28 days, since none were found in a large number of animals sacrificed in the 28th day of the experiment. In addition, Popper(8) could not demonstrate tumors before 51 days. Thus, it appears that during the 28-day period changes occurred which resulted in the later development of the nodules.

When nodules were found, after the relatively prolonged period of 63 days of ethionine feeding, the remainder of the liver showed active regeneration of parenchymal cells and marked bile duct proliferation in addition to fibrosis and chronic inflammation, whereas in the group that had been denied ethionine for the last 35 days of the experiment, the remaining liver had been restituted so that its architecture was essentially normal and no fibrosis was seen, although there was still a small amount of bile duct proliferation. These findings were the same in both the animals with liver nodules and those in which they were absent. This still does not answer the question as to whether ethionine has a carcinogenic action or whether the nodules develop in the course of regeneration.

Summary. The addition of 0.5% ethionine to the diet for 63 days caused the production of hepatic nodules resembling tumors in 16 out of 23 male albino rats. When ethionine was fed for 28 days and then removed for the remainder of the 63-day period, hepatic nodules were also produced in approximately the same proportion of cases (5 out of 8). No such nodules were observed in animals sacrificed after 28 days of ethionine administration.

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Gravitational Aspects of Growth as Demonstrated by Continual Centrifugation of the Common Fruit Fly Larvae.* (21869)

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One of the factors believed to influence growth of organisms is gravity. Experimental investigation of how development is altered by changing gravity has been quite limited. Matthews(1) reports that rats, which developed in a centrifugal field 3 times that of the earth's gravitational field, were smaller than normal rats. The purpose of this paper is to report that the growth of fly larvae was retarded by simulating an increased gravitational field.

Materials and methods. Newly hatched larvae of the common fruit fly (Drosophila melanogaster, wild type, red eye), cultured upon a banana-agar medium,† were subjected to continual centrifugation so as to produce the effect of increased gravity. They remained in a given centrifugal field throughout the experiment. The media was centrifuged under experimental conditions for 24 hours previous to the experiment and excess water, squeezed from the media during this process, was drained off. Control and experimental animals were placed on similar preparations.

Sizes of the larvae were recorded by means of contact photographs (Fig. 1). Volumes (v) were calculated by assuming the shape to be that of an ellipsoid of revolution so that the following equation would apply:

$$v = \frac{\pi w^2 l}{6}$$

where w is the width and l is the length. Growth constants (k's) were calculated by dividing the change in the natural logarithm of v by the time required for such a change. These changes were based upon 2 measurements of v, one made when the experiment began and the second, a day later. Approximately 20 test and 20 control animals were studied in each experiment. When no later records of size were desired, the animals were killed and fixed in a solution of formalin-acetic-alcohol. If experiments were continued, the larvae were photographed alive in water each day, placed on a fresh preparation of media, and returned to the centrifuge.

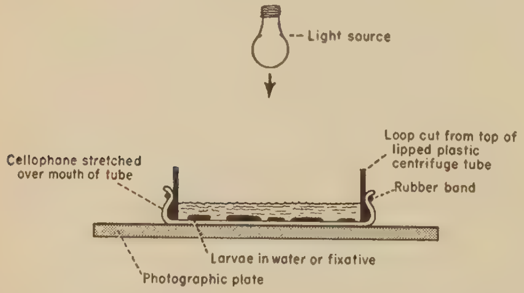
Light source
Cellophane stretched over mouth of tube
Loop cut from top of lipped plastic centrifuge tube
Rubber band
Larvae in water or fixative
Photographic plate

FIG. 1. Arrangement of materials used to record size of larvae by contact photographs.

Centrifugation of test preparations was carried out with a Servel, angle centrifuge (Type XL) in an incubator at 31.5 ± 0.3°C. Control preparations were placed in the same incubator. Field was varied in one of two ways: the rate of revolution could be controlled or the radius of revolution could be decreased by packing cotton in the bottom of the tube holders. In this paper, the field is expressed in g's, with one g equal to the Earth's gravitational field of 980 cm/sec^2.

Results. The growth rate was reduced by centrifugation (Fig. 2 and 3). In 3 different experiments, using 1630 g, 2250 g, and 3100 g, where the larvae were permitted to develop for several days, size leveled off before the size of normal larvae of pupation size could be attained. These larvae died without pupating. Both the decrease in growth rate and the decrease in size become more marked as the field increases.

Of the many factors which could be responsible for these decreases, probably the most important one is the increase of the

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* This work was supported in part by The Central Scientific Fund, College of Medicine, State University of Iowa.

† This medium is described in Turtox Service Leaflet No. 15, General Biological Supply House, Chicago, Ill.

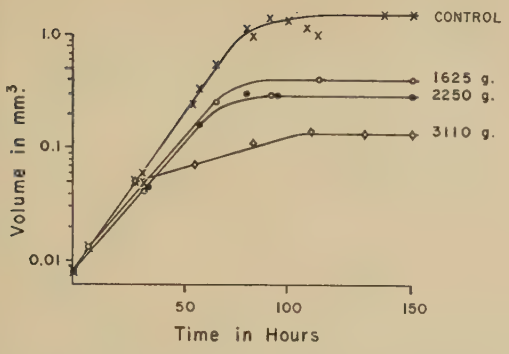

FIG. 2. Growth of drosophila larvae with different centrifugal fields. Experimental points represent logarithms of size (v) in mm³ as a function of time (t). These curves demonstrate that less growth is attained in the greater centrifugal fields. Final points represent average volume of control pupae or average size of experimental larvae at death.

weight with gravitational field for a body of constant mass. Since physical considerations show that the smaller a body is the better it can support its own weight(2), an animal would be expected to be smaller if it developed in a larger field. For growth to occur, food materials must come in contact with protoplasm. Before such contact is made, certain mechanical movements such as circulation, passage through the digestive tract, and locomotion are usually necessary. When the weight is increased, more effort is required in order to move the same amount of material. One of two things would be expected: energy which would otherwise be used for developmental processes would be diverted to overcome gravity, or less movement would occur. In either event, less growth occurs. The changes in development are no doubt a result of several factors.

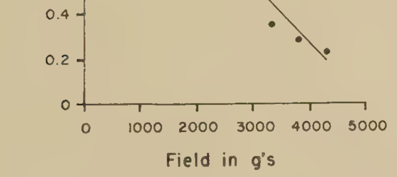

FIG. 3. Dependence of growth constant upon centrifugal field. Points represent experimental growth constant (K_x) relative to control constant (K_c) as a function of the field in g's. The curve is the best fitting straight line. Values for K_c were approximately equal to 0.07 hour^{-1} but they apparently depend to some extent upon condition of medium and initial size of larvae.

For the sake of simplicity, consider a hypothetical case where the principal influence upon growth rate results from the above mentioned diversion of energy to combat gravity. Assume that growing material can release energy, E_v, in proportion to its volume, and diverts energy, E_g, to overcome gravity in proportion to its volume and the field, g. Assume also that the rate of increase in volume (dv/dt) is proportional to the energy, E, available for growth. If E is the difference between E_v and E_g, then $dv/dt = c_1E = c_1 \; (E_v - E_g)$. However, $E_v = c_2v$ and $E_g = c_3vg$ where c_1, c_2, and c_3 are constants. So that $dv/dt = k_1v - k_2vg = (k_1 - k_2g)v$, where $k_1 = c_1c_2$ and $k_2 = c_1c_3$. Thus, $d(\log_e v)/dt = k_1 - k_2g = k$. This indicates a linear relationship between growth constant (k) and the field (g). This relationship overlooks such factors as heredity and food supply which also limit growth. Therefore, in its present degree of refinement, the hypothesis applies only to the early, or exponential, phase of development.

Values for the growth constant, k, as a function of field, g, have been fitted to a straight line by the method of least squares (Fig. 3). The equation for this line is similar to the above equation. $k_x/k_c = 1.33 \pm 0.015 - (2.69 \pm 0.04) \times 10^{-4}$ g. The fraction, k_x/k_c, is the experimental growth constant, k_x, relative to the control value, k_c. Quantities following the ± sign are the standard errors. More data will be necessary before it is possible to establish whether or not a truly linear relationship exists.

One unexplained result is that the value of k_x extrapolated to the earth's gravity of one g, which for these larvae is practically the same as zero gravity, does not correspond to the control value, k_c. In other words, the intercept, k_x/k_c, should be equal to unity rather than 1.3. Apparently, there are one or more secondary, experimental factors which do not act upon the control. Possible factors would include such things as centrifuge vibrations, undetected temperature differences, and orientation differences.

Summary. As the centrifugal field increases the size of fruit fly larvae becomes limited and the growth rate decreases. A possible explanation is suggested for the apparently linear decrease of growth rate with field.

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Competitive Binding of Calcium and Magnesium with Serum Albumin.* (21870)

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From the various studies of the binding of calcium and magnesium with proteins, it appears that these 2 ions behave very much alike in their formation of un-ionized complexes. In the few instances where direct comparisons have been made with individual proteins, little or no difference has been observed, either qualitatively or quantitatively. This includes the work of Greenberg and Schmidt(1) with casein, the work of Barany, Biro, and Molnar (2) with actin, and our own work with serum albumin, casein, pepsin, and lysozyme(3,4).

For calcium and magnesium complexes with non-protein substances, there is also a similar behavior except for some quantitative differences. In some instances the formation constant for the calcium complex is larger than for the magnesium complex, and in other instances the reverse is true. In many instances, however, these constants are nearly the same, and for at least one familiar substance, citric acid, the constants are identical (5).

In view of their very similar behavior in forming complexes, it is highly probable that calcium and magnesium ions are bound at the same sites on the proteins and would thus compete with each other. If the strength of the binding is about the same for both ions, it follows that the relative binding of the two ions would be in direct proportion to their concentrations. This idea of a possible competition between calcium and magnesium for proteins was first pointed out by Hastings and McLean(6). Using their formulation, we can express this relationship as follows:

$$\frac{(\mathrm{Ca}^{++})\,(\mathrm{Prot}^{=})}{(\mathrm{Ca\ Prot})} = \mathrm{K} = \frac{(\mathrm{Mg}^{++})\,(\mathrm{Prot}^{=})}{(\mathrm{Mg\ Prot})}$$

or

$$\frac{(\mathrm{Ca}^{++})}{(\mathrm{Mg}^{++})} = \frac{(\mathrm{Ca\ Prot})}{(\mathrm{Mg\ Prot})}$$

In order to test this relationship experimentally, we have measured the simultaneous binding of calcium and magnesium in solutions of bovine serum albumin.

Experimental. To determine the simultaneous binding of 2 ions, we have used the technic of equilibrium dialysis. This is a widely used method for studying the binding of ions to proteins, having been used especially by Klotz and co-workers for a large number of such measurements(7).

The protein used in these experiments was Armour's bovine serum albumin. Solutions of this protein were electrodialyzed just before use. The protein solutions were then adjusted to pH 7.4 by the addition of sodium hydroxide. Finally, known quantities of calcium and magnesium chloride solutions were added. A known volume of such a protein solution was placed inside a cellophane dialyzing membrane, and an equal volume of a calcium chloride-magnesium chloride solution

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TABLE I. Total Binding of Cations to Serum Albumin when Both Calcium and Magnesium Are Present. pH = 7.4.

Protein conc., %	Ratio Ca:Mg	R^2_{Cl}	$(Me^{++})_o$, mMol/l	$(\text{Total Me})_i$ mMol/l	$(Me^{++})_i$, mMol/l	Moles Me bound/10^5 g
1.0	1:0	1.04	6.42	7.22	6.67	5.5
1.0	1:1	1.04	6.64	7.47	6.90	5.7
1.0	0:1	1.04	6.52	7.38	6.80	5.8
2.0	1:0	1.08	6.13	7.88	6.62	6.3
2.0	3:1	1.08	6.12	7.83	6.61	6.1
2.0	1:1	1.08	6.16	7.83	6.66	5.9
2.0	1:3	1.08	6.18	7.83	6.68	5.8
2.0	0:1	1.08	6.17	7.95	6.67	6.4

was placed outside. The system was then allowed to equilibrate by standing at 3°C for 72 hours. Preliminary measurements showed that equilibrium was always attained in this time. The 2 compartments were then analyzed for their various constituents. In some of the experiments only total divalent cations and chloride were determined; in others calcium, magnesium, and chloride were determined.

The analysis for chloride was made by an amperometric titration(8). Total divalent cation was determined by titration with ethylene-diamine tetraacetic acid according to Sobel and Hanoch(9). Calcium was determined by the method of Clark and Collip (10), and magnesium was determined by precipitation with 8-hydroxyquinoline and subsequent determination of the amount of precipitate by means of an amperometric titration(11). Where comparisons were made, the calcium and magnesium determinations were in agreement with the total divalent cation determinations.

Results. In our first set of experiments the ratio of calcium to magnesium has been varied over a wide range, while the total cation concentration was kept constant. The results of these experiments are presented in Table I. In column 2 is given the ratio of calcium to magnesium in the protein solution as it was first set up. In column 3 is given the square of the ratio of the chloride concentration outside to the chloride concentration inside at equilibrium. In column 4 is given the total concentration of calcium and magnesium in the solution containing no protein (outside solution), and in column 5 is given the same concentration for the solution containing the protein (inside solution). In the sixth column are listed the calculated values for the concentrations of ionized calcium and magnesium in the protein solutions. These calculations are made according to the principles of the Gibbs-Donnan equilibrium. On the basis of other experiments(12) the binding of chloride to serum albumin is taken to be insignificant under these conditions. In addition it is assumed that there is no significant difference in the activity coefficients for a given ion on the 2 sides of the membrane. Finally, the degree of binding is calculated by taking the difference between the total concentration of cations inside (column 5) and the concentration of free cations inside (column 6) and dividing by the concentration of protein. According to results in Table I, it is seen that there is no significant difference in the total cation binding, regardless of the relative concentrations of the 2 ions.

To test this idea further, other experiments have been carried out in which more complete analyses were made. In these instances the calcium and magnesium concentrations were determined separately. A summary of several typical experiments is given in Table II. In columns 3 and 4 are the measured concentrations of calcium in the outside and inside solutions, and in columns 5 and 6 are the same values for magnesium. In column 7 is the ratio of the concentrations of free ion in the protein solution, and in column 8 is the ratio of the amounts of each ion bound to the protein. It will be seen that for each case within the experimental error, the ratio $(Ca^{++})_i/(Mg^{++})_i$ is the same as (Ca Prot)/(Mg Prot).

Discussion. The foregoing results show

TABLE II. Simultaneous Binding of Calcium and Magnesium to Serum Albumin. $pH = 7.4$.

Protein conc., %	R^2_{Cl}	$(Ca)_i$, mMol/l	$(Ca)_o$, mMol/l	$(Mg)_i$, mMol/l	$(Mg)_o$, mMol/l	$R_{(Me^{++})i}$	R_{MeProt}
3.0	1.16	7.77	5.25	4.06	2.85	1.8	2.3
3.9	1.03*	3.18	2.13	1.80	1.27	1.7	2.0
2.0	1.09	2.50	1.93	2.50	1.95	1.0	1.1
2.0	1.06	2.46	1.93	2.48	1.97	1.0	1.1
3.0	1.16	3.88	2.73	7.83	5.43	.50	.46
1.9	1.08	2.00	1.56	5.83	4.62	.34	.38

* The NaCl concentration was 0.1 *M*.

quite definitely that calcium and magnesium compete with each other on an equal basis for combination with serum albumin. This situation was predicted from earlier results which showed that the binding of magnesium to serum albumin followed the same pattern as the binding of calcium when the 2 ions were present by themselves in solutions of serum albumin. Similar earlier results also showed that calcium and magnesium were bound to serum globulins, casein, egg albumin, pepsin, and lysozyme to the same extent. Thus it might be easily inferred that when calcium and magnesium are present simultaneously in a solution of any of the above proteins, the relative binding of these 2 ions would also be directly proportional to their relative concentrations. This does not imply, however, that the binding of calcium and magnesium is necessarily the same with all proteins. It is still quite possible that there are proteins not yet studied which would show a difference in their binding between these 2 ions.

In the comparison of these results with the situation in serum, the picture is not completely clear. Most of the studies of the binding of these ions in serum indicate that the percentage of bound calcium is higher than the percentage of bound magnesium. It is generally agreed that calcium is bound to the extent of about 50% in normal serum. For magnesium it appears that about 35% of it is bound to the serum proteins. This value is somewhat in doubt, however, because there has been considerable variation in the values reported for bound magnesium(13). At least one report, that of Greene and Power(14), indicated that the percentage binding of calcium and magnesium was approximately the same. In addition, because serum is composed of many substances besides proteins, it is quite possible that the percentage of diffusible calcium and magnesium could be different even though the binding relationships with protein were the same.

Whether or not there is a quantitative difference in the percentage of calcium and magnesium bound in serum, it should be pointed out that a considerable amount of indirect evidence has accumulated which indicates that there is calcium-magnesium competition in serum. In many instances when the calcium level is raised *in vivo,* the magnesium level falls and vice versa. For example, Mendel and Benedict(15) reported that parenteral administration of magnesium salts in dogs and rabbits resulted in an increased urinary output of calcium. In the same way the intake of calcium resulted in an increased excretion of magnesium. Many reports since that time have confirmed this general effect (16). On the basis of competitive binding with proteins, this behavior would be expected. For example, an increased intake of calcium would decrease the amount of magnesium bound to proteins which would cause an increased concentration of ionized magnesium in the plasma. The tendency towards an increased concentration of ionized magnesium would thereby lead to an increased excretion of magnesium. This picture is consistent with the idea that the physiological regulation of the blood level of calcium and magnesium is dependent only on the ionized forms of these substances. The amount bound by the proteins depends only on the mass action effect on the interaction of these ions with the various proteins of the plasma.

In view of this competition between calcium and magnesium for binding with proteins, it would seem that when ionized calcium is being determined, the concentration of magnesium should also be known. From the practical standpoint, this is probably not very important because the normal magnesium level is only about one-half that of the calcium, and in addition it very rarely changes to any extent. On the other hand, calcium changes are more frequently observed, and because of its greater concentration, these changes could markedly alter the magnesium binding. Thus it would appear to be essential at all times to take the serum calcium into account whenever the magnesium level is being determined.

Summary. A study has been made of the binding of calcium and magnesium to serum albumin when both are present at the same time. It is shown that the ratio of the concentrations of bound calcium and bound magnesium is equal to the ratio of the concentrations of the ionized calcium and ionized magnesium.

The author wishes to acknowledge the technical assistance of Warren J. Warwick.

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Preservation of Cell Cultures at 4°C.* (21871)

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Studies with cell cultures of mammalian origin which are maintained by constant serial passage are hampered by the possibility of loss by microbial contamination and the inevitable laboratory mischances. Also, serious limitations are imposed by the time and materials required for the maintenance of cultures not actively under investigation. Fischer (1) attempted to circumvent some of these difficulties in plasma cultures of chick embryo by lowering the temperature of incubation to 20°C, thereby reducing the required frequency of fluid replacement and subculture. Other investigators(2,3) have successfully employed similar procedures with a variety of tissues, but it has been observed that cultures placed at 0°C for short periods of time fail to survive(1,4). On the other hand, investigators have frequently taken advantage of the fact that fragments of fresh tissue remain viable for protracted periods at temperatures of 0-15°C(5). This report describes the storage of stable cell strains at 4°C for prolonged periods without replacement of the nutrient fluid.

Materials and methods. Cells. The cell

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* Aided by a grant from the National Foundation for Infantile Paralysis.

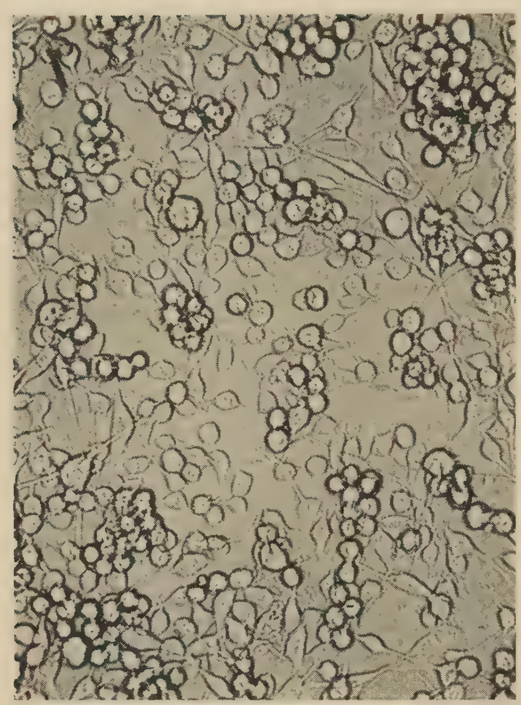

FIG. 1. Cells of strain L, growing on glass.

lines studied include strain L of Earle(6), strain HeLa of Gey(7) from an epidermoid carcinoma of the cervix and strain MB 13 isolated originally from a mouse lymphosarcoma by de Bruyn(8). In addition, a number of cultures established in this laboratory which have been serially propagated for periods exceeding 18 months are included in this study: CM2 isolated from a mixture of skin, subcutaneous tissue and muscle of a 7-day chick embryo; FS4 and U12 derived from human foreskin and uterus respectively. These cultures resemble the L strain in many respects. All have the appearance of fibroblasts (Fig. 1), grow readily on a glass substrate predominantly as single cells and are readily dislodged from the glass by mechanical procedures to yield suspensions composed primarily of single cells or small clumps of cells.

Preparation of Embryo Extract. Chick embryo extract (CEE50) is prepared essentially by the method of Bryant, Earle and Peppers(9). Embryos of 14-day incubation at 38.5°C are harvested aseptically, and processed at 4°C. The embryo paste obtained by homogenization in a Waring blender for 1.5 minutes is frozen and then allowed to thaw slowly in the refrigerator. An equal volume of Earle's balanced saline(10) lacking sodium bicarbonate is added, the pH adjusted to 7.0-7.4 with sodium hydroxide and the mixture allowed to stand for 24 hours with occasional mixing. The larger tissue fragments are removed by centrifugation at 1000 x g for 45 minutes. Three mg of hyaluronidase (Worthington, 200-250 TRU/mg) are added per 100 ml of pooled supernate and the solution is incubated at 37°C (water bath) for 3 hours. The hyaluronidase-treated extract is then centrifuged at 35,000 x g for one hour (Spinco preparative centrifuge). The supernate is filtered through a Selas filter of 03 porosity and stored at –20°C.

Beef embryo extract (BEE50) is prepared from embryos weighing from 0.5 to 2.5 kg. The whole embryo is used except that the large bones and hooves are removed from those weighing more than 1.5 kg. Intact uteri are obtained from a local abattoir as soon as they are removed from the animal and the embryos are processed within a few hours. The embryos are passed through a tissue grinder fitted with a grinding plate of 2 mm porosity. BEE50 is prepared from the resulting embryo paste in the manner described for the preparation of CEE50.

Nutrient Fluids. The composition of the media employed for the propagation of cells and their designation in this laboratory are indicated in Table I. All nutrient fluids are prepared in the quantities required for a period of 4 to 6 weeks. Each lot of fluid is sterilized by filtration through an 02 Selas filter and stored at 4°C.

TABLE I. Composition of Cell Culture Media.

Medium	Composition* (v/v)							
	%			%			%	
CF	5	CEE50	+	10	NHS	+	85	FS
F17	10	”	+	20	”	+	70	”
705	5	”	+	20	”	+	75	703
715	5	BEE50	+	25	HUS	+	70	”
E7	5	CEE50	+	40	NHS	+	55	ES

* The following abbreviations are employed: NHS = normal horse serum; HUS = pooled human serum after treatment with zymosan(13), kindly supplied by Dr. L. Pillemer; FS = V-614 mixture of Fischer *et al.*(11); 703 = mixture 703 of Healy, *et al.*(12); ES = Earle's balanced saline (10).

TABLE II. Survival Time of Cells Stored at 4°C.

Culture	Medium	Cells/cm² × 10^5*	Survival time (wk)
CM2	F17	1.5	6
FS4	705	2.9	6
HeLa	715	2.1	7
L	CF	.9	9
L	E7	.9	6
MB13	E7	3.9	6
U12	705	4.3	6

* Numbers of cells when cultures were placed at 4°C are expressed as cells per cm² of glass surface since several types of tissue culture flasks were employed.

Propagation of cells. It is important that cell cultures be propagated under standardized conditions, otherwise wide variations in response to test conditions may be observed. All cultures are transferred at weekly intervals and are employed for experiments between the 6th and 8th day. Subcultures of CM2, FS4, L, MB13 and U12 are prepared as follows. The cells are dislodged from the surface of the flask with a rubber policeman and withdrawn from the flask together with the medium. Three volumes of fresh fluid are added, the cell clumps are largely dispersed by repeated and vigorous pipetting, and the cells are counted in a hemocytometer chamber. In preparing replicate cultures for experiments where greater accuracy is required a suspension of single cells is readily obtained by filtration through sintered glass (Corning, porosity B). The density of the cell suspension is adjusted to 10^5 cells per ml and added to fresh flasks (0.16 ml per cm² surface). Strain HeLa is subcultured similarly except that the cells are removed from the glass by treating with 0.025% trypsin (Nutritional Biochemicals Corp., 2 x crystallized containing 50% $MgSO_4$) in Mixture 703(12), pH 8.0-8.2, for 10 minutes at 37°C. The nutrient fluids are replaced on the third and fifth day in all cultures except strain HeLa where the fluid is replaced daily after the first 48-hour interval.

Experiments and results. For each experiment a number of replicate cultures were prepared and allowed to grow in the usual manner until the bottom of the flasks was covered with a continuous layer of cells, usually 7 days. The cells were then stored at 4°C in the medium employed for growth. The fluid was changed at the time the cultures were refrigerated except in a few instances the medium was replaced 24 hours prior to storage. No further replacements were made for the duration of the refrigeration interval. Flasks were removed from 4°C and placed at 37°C each week until the cultures were no longer viable as indicated by their inability to proliferate. After 2 to 3 days at 37°C one-half of the nutrient fluid was replaced with fresh medium. In 5 to 6 days all the fluid was replaced and those cells floating free were discarded. The time of fluid changes is dependent on the rate of recovery of the culture and is related to the cell strain and the storage time. The data presented in Table II summarize the results obtained in a number of experiments with each cell strain.

Within 2 or 3 days after cultures are placed in the refrigerator a large proportion of the cells tends to contract, cell processes to withdraw and the cells assume a spherical shape (Fig. 2). There are no apparent changes in the course of the next 2 months. When flasks are withdrawn from storage and placed at

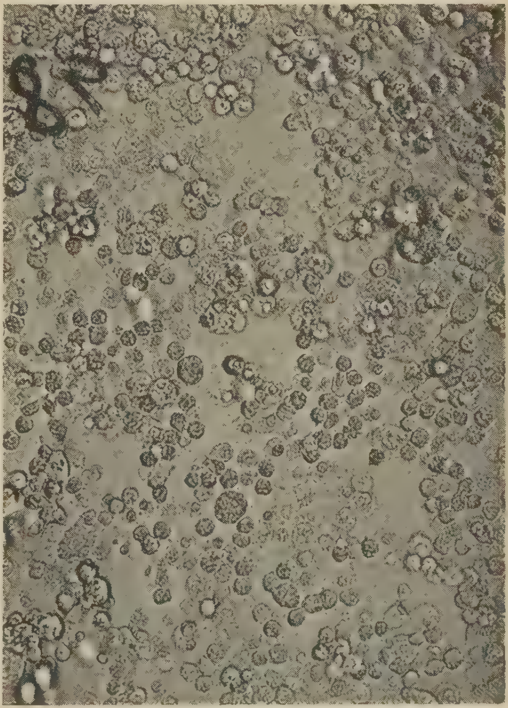

FIG. 2. Cells of strain L, 24 hr after being placed at 4°C.

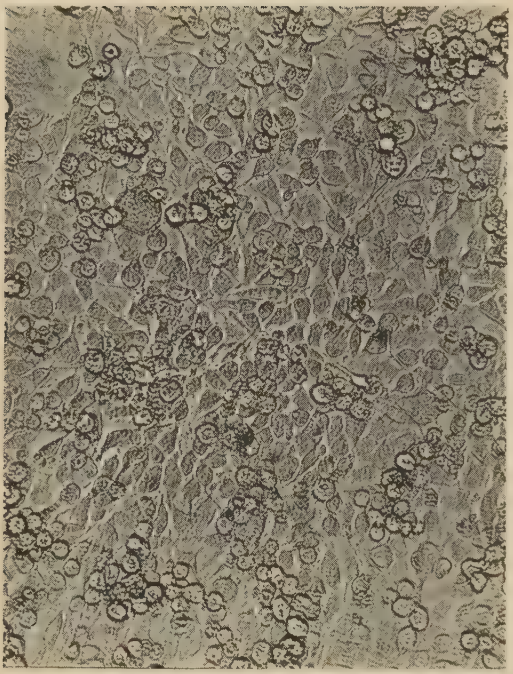

FIG. 3. Cells of strain L, 4 days after transfer from refrigerator (1 mo) to 37°C.

37°C without replacing the medium, the cells gradually extend their processes and in a few days assume the form characteristic of the strain (Fig. 3) and begin to multiply. Accurate counts of surviving cells have not been made but it appears that a considerable proportion of the cells fail to survive refrigeration. If refrigeration of a culture is to be continued, best results are obtained if the cells are first subcultured before they are returned to 4°C. For example, many of the strains listed in Table II have been maintained for the past 6 months on a 7-week cycle: 5 weeks at 4°C, one week for recovery followed by subculturing to fresh flasks, and the seventh week to grow a new crop of cells before returning to the refrigerator. Thus, active care of these cultures is required for only 2 out of every 7 weeks.

Discussion. It has been found essential for prolonged storage that the cells be in good condition upon refrigeration, and that no fluid changes be made during the storage period and for 2-3 days after their return to 37°C. In spite of the fact that survival is prolonged in the absence of fluid replacements, the cells continue to metabolize slowly at 4°C as indicated by the production of acid.

Freshly established fibroblast cultures derived from human tissues fail to survive relatively short periods of refrigeration. The results of earlier investigations(1,4) indicate that cells comprising fragments of tissue withstand refrigeration more readily than actively growing cells of a young culture. It is not apparent whether cell state alone is the controlling factor or whether cell state at the time of refrigeration together with the character of the fluid in which the cells are maintained determines the outcome. The results of preliminary experiments with some of the cultures listed in Table II indicate that the period of satisfactory storage may be prolonged by alterations in the fluid phase.

While these studies were in progress Scherer and Hoogasian(14) reported that cultures of strain L and HeLa could be preserved for at least 6 months by applying the technic of freezing cells in dilute glycerol solutions. This method of cell storage will undoubtedly prove increasingly useful in studies on the stability of cell lines under various cultural conditions and for the preservation of cultures. On the other hand, storage at 4°C satisfies the need in the tissue culture laboratory for a method whereby cells may be maintained with a minimum of handling and is a simple technic providing considerable insurance against loss of cultures by microbial contamination and other accidents.

Summary. A method is described whereby stable strains of cultured cells may be preserved at 4°C for periods in excess of 6 weeks. If sufficient time is allowed for recovery of cultures the process may be repeated many times. Essentials of the method are that the cells be in good condition when refrigerated and that no replacement in the nutrient fluid be made during the storage period and for several days after the cells are returned to 37°C.

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Free Amino Acids of Muscle of Normal and of Vitamin E-Deficient Rabbits. (21872)

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In many species, though not in man, a deficiency of vit. E results in a muscular wasting and weakness that bears a marked resemblance to human progressive muscular dystrophy. Alterations that may be demonstrated in the muscle substance during the course of the nutritional disease include an increased oxygen consumption(1), an increase in the relative collagen content(2), a decreased loss of amino nitrogen from slices *in vitro*(3), and an increase in the proteolytic activity(4). In the human disease, there is in the muscle a similar increase in the ratio of the collagenous nitrogen to total protein nitrogen, and also decreases in the contents of certain enzymes (phosphorylase a, phosphorylase b, aldolase, and phosphoglucomutase), as well as a decrease in overall glycogenolysis (5,6). It was of interest to determine whether changes in the proteins of the muscle, indicated by the above findings, are reflected in changes in the free amino acids of the muscle tissue.

Methods. Through the cooperation of Dr. Anne E. Milman,* litter-mate rabbits were maintained on either a vit. E-deficient or an adequate diet, and were pair-fed, as described by Milman and Milhorat(7). The onset of dystrophy in the deficient animals was considered as the point at which the creatine excretion rose markedly. On this basis, animal K-24 (Table I) was just barely dystrophic, while animal J-6 was in an advanced stage of dystrophy and showed signs of weakness and loss of muscular function. Animals K-25 and J-9 were respective normal littermates.

Prior to the estimation of amino acids, the animals were anesthetized with intravenously-administered amytal and were exsanguinated. Both gastrocnemii were removed and deproteinized with picric acid by the method of Hamilton(8) as previously described(9). The deproteinized extracts were analyzed for amino acids and related substances by chromatography on Dowex 50-X4 (10). Calculation of the amounts of 1-methylhistidine, carnosine, and anserine required chromatographic analyses of hydrolysates of each extract, in order to determine the histidine, β-alanine, and 1-methylhistidine present after acid hydrolysis, as previously outlined(9). As a check on these calculations, the ninhydrin color values of the 3 components were computed, totalled, and compared to that observed in the single peak containing anserine, carnosine, and 1-methylhistidine on the chromatogram of the unhydrolyzed extract. The observed and calculated figures

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* We are greatly indebted to Dr. Anne E. Milman, of the Department of Psychiatry, Cornell University Medical College, and The New York Hospital, for the tissues from the normal and deficient animals which have been analyzed in this study.

TABLE I. Amino Acids and Related Compounds in Muscle of Normal and of Vit. E-Deficient (Dystrophic) Rabbits. Values as mg per 100 g, wet wt, of tissue.

	Animal				Literature values		
	K-25	K-24	J-9	J-6	Normal rabbits		
Constituent	Normal	Early dystrophy	Normal	Severe dystrophy	Dubreuil and Timiras (11)	Duchâteau and Florkin (12)	Krueger (13)
Aspartic acid	1.9	3.4	2.2	15.0	.5	2.5	
Glutamic acid	18.4	16.5	16.9	62.4	11.3	19.3	
Glycine*	64.9	18.2	53.6	29.0	33.4	40.9	18.2
Alanine	5.6	14.0	9.3	20.5	6.9		
β-Alanine	3.4	15.7	1.0	1.5	+		
Valine	1.6	4.7	4.0	5.0	1.6	4.3	
Leucine	1.0	2.9	1.4	4.4	3.9	3.5	
Isoleucine	1.4	2.6	1.4	2.6		2.7	
Serine	8.4	4.6	9.0	12.4	4.3		
Threonine	1.4	4.2	4.2	10.0	2.0	3.8	
Methionine	.3	.4	.4	.9	+ (?)	2.2	
Cystine	0	0	0	3.2			
Taurine	66.8	62.8†	43.7	40.2	3.0		
Glutathione‡	28.3	47.6	36.9	44.1			
Proline	L.§	L.	13.3	21.2	4.5 (?)	6.8	
Phenylalanine	.7	1.0	.5	1.8		2.1	
Tyrosine	.6	2.4	1.8	3.1	1.7	2.3	
Histidine	1.2	2.6	6.6	3.1		3.7	
3-Methylhistidine	L.	1.0	.7	1.1			
1-Methylhistidine‖			18	1			
Carnosine‖			46	20	+		
Anserine‖			194	58			
Ornithine	1.7	1.1	3.1	3.4			
Lysine	1.8	3.5	6.1	5.5	3.0 (?)	4.4	
Arginine	1.8	1.5	4.2	3.7	4.2 (?)	3.5	
Urea	24.3	45.5	42.5	65.7			
Ethanolamine	4.9	3.9	1.4	1.0			
Phosphoethanolamine	4.1	L.	4.7	34.1			
Glycerophosphoethanolamine	2.0	9.8	1.4	14.2			

* Contains citrulline; see text.
† " phosphoethanolamine, which did not separate.
‡ " aminoadipic acid and α-amino-n-butyric acid(10).
§ L. indicates that determination was lost.
‖ For calculation, see text.

agreed to within ± 2%.

In view of the lower values for glycine in normal rabbit muscle (Table I) reported by other workers(11-13), the contents of the glycine peak in the present experiments were investigated further. A portion of the extract from the muscle of normal rabbit K-25 was chromatographed on a 150 cm column of Dowex 50-X4 at 50° with pH 3.1 buffer(10) to reveal the presence of any citrulline, which appears with glycine under the usual chromatographic conditions. Citrulline was present to the extent of only 4 mg%. The effluent fractions that contained the glycine peak were desalted by the procedure of Drèze *et al.*(14), and half of the desalted material was hydrolyzed with 6 N HCl in the usual manner. After removal of the HCl from the hydrolysate, both hydrolyzed and unhydrolyzed materials were chromatographed on paper (Whatman No. 1, ascending technic) with a solvent composed of *sec*-butyl alcohol(75 ml), water (10 ml), and 88% formic acid (15 ml) (15). The unhydrolyzed material, glycine, and a mixture of the 2, travelled as single spots with the same R_f value (0.22); the hydrolyzed material and a mixture with glycine travelled as single spots of R_f 0.19, having been slightly retarded by a salt effect. A faint spot remained at the starting point with the unhydrolyzed material and there was a faint spot of R_f 0.04 with the hydrolyzed material.

Results and discussion. The concentrations of the free amino acids and related compounds in the muscle extracts are given in Table I, together with previously reported values for normal rabbits. It will be noted from the Table that in the severely dystrophic animal (J-6) there is a general increase in the concentration of the free amino acids, involving all of those found in proteins with the exception of glycine and the basic amino acids. This observation may be reconciled with losses in muscle protein, but it does not in itself support a concept of an increased catabolism of the proteins; a decreased anabolism might equally well cause an accumulation of free amino acids. Phosphoethanolamine, glycerophosphoethanolamine, and urea also rise in concentration, but the other compounds and the amino acids which are not constituents of protein either remain constant or fall off in concentration. Glutamine, which is not determined quantitatively by the present procedure(10), has been found by Roderuck(16) to be decreased in concentration in the muscle of dystrophic rabbits.

Running counter to the general increase in amino acids is the marked decrease in the concentration of glycine, even at the earliest stage of dystrophy. Though the glycine peak on the chromatogram contains citrulline, as well as a trace of an unidentified compound, the change noted here is of so great an extent that it must be primarily a result of a decrease in the level of glycine. This decrease is greater than the decrease in non-collagen protein N found by Spencer *et al.*(2) with similar material, and would not be eliminated if the results were expressed in terms of non-collagen protein N (cf. Lilienthal *et al.*(17)). A similar decrease in the concentration of glycine in the muscle of scorbutic guinea pigs has been noted by Christensen and Lynch(18), who point out that this decrease may be related to changes in the metabolism of collagen.

Another marked change is the reduced concentration of 1-methylhistidine, carnosine, and anserine in the muscle of the severely dystrophic animal. A comparison of animals K-25 and K-24 showed that the early dystrophy was not accompanied by a decrease in the size of the combined 1-methylhistidine, carnosine, and anserine peak. The concentrations of the individual substances were not determined in this instance. A decrease in anserine and carnosine in dystrophic animals has been noted previously by Shabanova(19).

Summary. 1. The concentration of free amino acids and related compounds in muscle extracts from normal and from vit. E-deficient (dystrophic) rabbits has been determined by ion-exchange chromatography. 2. In the severely dystrophic animal, the levels of most of the amino acids were elevated. 3. 1-Methylhistidine, carnosine, and anserine were decreased in concentration. 4. The concentration of glycine was markedly reduced, even at the earliest stage of the disease.

The author wishes to acknowledge the technical assistance of Miss Renate Mikk in the performance of the chromatographic experiments.

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Propagation and Primary Isolation of Mumps Virus in Tissue Culture.* (21873)

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Cytopathogenic effects of mumps virus have been demonstrated in tissue cultures of human cancer cells (strain HeLa) and of monkey kidney epithelium(1). Mainly cytolytic lesions were observed following inoculation of several strains, which had been adapted to propagation in the allantois of the chick embryo. However, these lesions were noted only when large amounts of virus were inoculated and cytolysis was no longer seen on second or third passages. Thus it was evident that these strains failed to propagate in these tissue cultures. In subsequent experiments, to be reported here, strains of mumps virus were employed which had been isolated from patients in the amnion of chick embryos and which had undergone only few serial amniotic passages. These produced similar lesions in tissue cultures, even when inoculated in high dilution, and could be maintained through many serial passages in HeLa or monkey kidney cells. Furthermore, primary isolations of mumps virus were readily achieved in these cultures.

Methods and materials. Virus. The Tucker, DD and Po strains were used. These had been isolated in the amnion of the chick embryo and amniotic fluids of the 1st to 6th passages had been preserved in sealed ampuls in the dry ice chest. Saliva was collected from acute cases of parotitis, contacts of mumps patients and from normal individuals. When possible, the specimens were brought directly to the laboratory, packed in ice, and prepared and inoculated immediately. Other samples were stored at –10 to –15°C. Before inoculation the specimens were clarified at 2000 rpm for 10 minutes and incubated at 37°C for 1 hour in the presence of 500 units of penicillin and 500 μg of streptomycin per ml.

Tissue culture technics. HeLa cells were grown and maintained as described previously (1). They were inoculated usually 3-4 days after seeding of approximately 60,000 cells per tube with 0.2 ml of the virus preparations. For convenience, a maintenance medium was used which consisted of 10% horse serum; 60% Earle's solution containing 2% parenamine; and 30% medium #199. Under these conditions the cultures could be maintained without feeding for 6-8 days. Cultures of monkey kidney epithelium were received from the Microbiological Associates, Inc. They were inoculated 3-5 days after seeding of 300,000 cells per tube and maintained on Medium #199 containing 5% horse serum. In some experiments monkey kidney cells were used which were derived from passage of cells grown in bottles for approximately 7 days.

Isolation of virus in chick embryos. Seven to 8-day-old chick embryos were inoculated with 0.2 ml of saliva by the amniotic route, as previously described(2). After 6 days of incubation at 35-36°C, the amniotic fluids were harvested individually under aseptic conditions and tested for hemagglutinins with 10% or 1% chicken red cells by the slide or test tube technics, respectively. Eight to 10 chick embryos were inoculated and a specimen was recorded as negative only when no hemagglutinins were found after 3 amniotic passages.

Experimental. Propagation of amniotic strains of mumps virus in tissue culture. Upon inoculation of amniotic fluids infected with recently isolated mumps virus cytolytic as well as degenerative lesions were observed in HeLa and monkey kidney cells, when the inoculum was diluted as high as 10^{-5}. The results of a representative titration of the Po strain in HeLa cells are shown in Table I. Titrations in monkey kidney cells gave comparable results except that the lesions tended

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TABLE I. Titration of Strain Po in HeLa Cells (Amniotic Fluid of the Third Egg Passage).

Virus dilution	Cytopathogenic effect Days of observation 2	3	4	6	8
10^{-1}	1*	4	4	4	4
10^{-2}	±	1	2	2	4
10^{-3}	0	0	±	1	2
10^{-4}	0	0	0	±	1
10^{-5}	0	0	0	0	0

* Lesions were graded according to estimated percentage of cells affected; 4 = about 100%; 3 = 75%; 2 = 50%; etc.

to become detectable somewhat later. These observations were in contrast to those recorded with allantoically-adapted strains, which gave titration endpoints of at most 1:32. Furthermore, the lesions produced by the latter agents appeared early and remained stationary after 24-48 hours of incubation, whereas those induced by the amniotic strains became detectable with increasing delay and progressed, often to complete destruction of the cultures, in the course of 8 days depending on the concentration of virus in the inoculum. At the end of the incubation period the uninfected control and the partially destroyed infected cultures started to degenerate on account of overcrowding. When cells from the latter preparations were reseeded into several new test tubes they continued to propagate for a short period of time, but then typical mumps lesions developed which progressed ultimately to complete destruction of the cultures.

The above data indicated that mumps strains maintained in the chick embryo for only a few passages retained their capacity to propagate in human or simian cells. This was further established by serial passages of the 3 strains employed in HeLa or monkey kidney cell cultures for up to 20 transfers at present. The medium, or the medium as well as the cells were collected (when cytolytic lesions had become apparent) and transferred to new cultures in dilutions of not more than 10^{-2}, since the infectivity endpoints in tube cultures rarely exceded this value. In bottle cultures, which contained about 2 million cells, titers up to 10^{-4} could be attained. The titers thus far have not increased on serial passage in these types of cultures. No hemagglutinins for human, chicken, guinea pig or sheep erythrocytes became detectable under these various conditions except in infected bottle cultures of monkey kidney epithelium, where titers up to 1:16 were noted.

Primary isolation of mumps virus in tissue culture. The results obtained with the amniotic strains suggested the possibility of primary isolation of virus from mumps patients in tissue culture. Correspondingly, salivas were collected from 29 children with parotitis, from 3 contacts and from several unexposed healthy persons. Eleven of the mumps specimens were inoculated into HeLa cell cultures and chick embryos on the day of collection without prior freezing. The other 21 specimens were kept frozen at −10 to −15°C for 1-4 days before testing. Isolation of virus from the fresh specimens, taken on the day of onset of illness or up to 4 days later, with one exception gave positive results in HeLa cells in the first passage (Table II). It is remarkable that significant lesions of varying extents were observed with 2 of the specimens after 24 hours, with 3 on the 2nd and with the remaining 5 on the 3rd day of incubation. The 11th specimen taken only on the 4th day of illness (Case #31) failed to reveal virus even after 3 serial transfers. In contrast, isolation of virus in the chick embryo was less successful. Of the specimens which yielded virus in HeLa cells, 2 failed to do so in the amnion, 3 produced detectable hemagglutinins in the first, 3 in the 2nd and one only in the 3rd passage. Specimen #31, which failed to yield virus in HeLa cells, likewise was negative in the chick embryo. Since the chick embryos were incubated for 6 days in each passage, the results were obtained with considerable delay as compared to the tissue culture technic. Three of these specimens were also tested in monkey kidney cells. Lytic lesions, resembling giant cells, were found in two of these after 6 and 4 days, respectively, and specimen #31 was negative also in this type of culture.

Isolation of virus from specimens which were stored at −10 to −15°C prior to inoculation were less successful. Rapid freezing and storage at dry ice temperature was unfortunately not possible under the conditions the specimens were obtained. It can be seen in the lower part of Table II that again the in-

TABLE II. Isolation of Mumps Virus from Acute Cases of Parotitis.

			Technic								
	Saliva		Chick embryo hemagglutination		HeLa cells cytopathogenicity		Monkey kidney cytopathogenicity		Comparison of results		
Case No.	Days after onset	Frozen	Pass. No.	Total days	Pass. No.	Total days	Pass. No.	Total days	Chick embryo	HeLa	Monkey kidney
12	0	No	3	18	1	2	×	×	+	+	
33	0	”	×	×	1	3	×	×	×	+	
6	1	”	2	12	1	2	×	×	+	+	
7	1	”	2	12	1	2	×	×	+	+	
8	1	”	1	6	1	1	×	×	+	+	
9	1	”	1	6	1	1	×	×	+	+	
10	1	”	1	6	1	3	×	×	+	+	
11	2	”	—	—	1	3	×	×	—	+	
14	2	”	2	12	1	3	1	6	+	+	+
30	2	”	—	—	1	3	1	4	—	+	+
31	4	”	—	—	—	—	—	—	—	—	—
Total (11)									7/10	10/11	2/3
4	0	Yes	—	—	1	4	×	×	—	+	×
5	0	”	2	12	Herpes	?	×	×	+	?	×
15A	0	”	2	12	—	—	—	—	+	—	—
20	0	”	2	12	2	9	2	11	+	+	+
21	0	”	—	—	2	11	2	15	—	+	+
24	0	”	2	12	1	2	1	4	+	+	+
16	0	”	—	—	—	—	—		—	—	—
34	0	”	—	—	1	4	×	×	—	+	×
32A	0	”	2	12	2	13	×	×	+	+	×
32B	1	”	×	×	2	14	×	×	×	+	×
2	1	”	—	—	1	4	×	×	—	+	×
3	1	”	—	—	—	—	×	×	—	—	×
17	1	”	2	12	1	3	1	6	+	+	+
26	1	”	—	—	—	—	—	—	—	—	—
22	2	”	—	—	—	—	1	5	—	—	+
25	2	”	—	—	—	—	—	—	—	—	—
28	2	”	3	18	1	5	1	5	+	+	+
15B	3	”	—	—	—	—	—	—	—	—	—
Total (18)									7/17	10/18	6/11
23	Contact	Yes	—	—	1	2	1	5	—	+	+
27	”	”	—	—	—	—	—	—	—	—	—
1	”	”	—	—	—	—	—	—	—	—	—

× = not tested; — = negative in 3 consecutive passages.

cidence of isolations was higher and the results were obtained sooner in HeLa cells than in the chick embryo. However, in 2 instances virus was isolated in the amnion but not in HeLa cells. In one of these (case #5) a strain of herpes simplex virus was obtained which destroyed all the cells in 3 days and thus possibly interfered with the demonstration of mumps virus. In the other instance (case #15A) the culture was probably damaged when the incubator temperature rose accidentally to 101°F for at least 12 hours. This accident may also have been responsible for the fact that virus became detectable in some instances only on 2nd passage after total incubation periods of from 9 to 14 days, or not at all. On the other hand, 5 specimens of saliva yielded mumps virus in HeLa cells but not in the chick embryo. The results in monkey kidney epithelium agreed with those recorded in HeLa cells with one exception (case #22). In this instance the latter failed to give evidence of infection. Saliva of one of the 3 contacts of mumps patients yielded mumps virus in HeLa and monkey kidney cells but not in the chick embryo (Table II). None of the salivas from 6 healthy, unexposed individuals produced cytopathogenic effects.

The viruses isolated in the chick embryo were identified by hemagglutination inhibition tests with specific mumps immune sera obtained from guinea pigs following intraperitoneal injection of mumps virus. None of the tissue culture strains gave evidence of hemagglutination as yet with human, guinea pig, chicken or sheep erythrocytes. Identification of the isolated agents was achieved therefore with representative strains only by neutralization tests in tissue culture with paired human sera obtained in the early acute stage of illness and during convalescence. In each instance the acute-stage serum revealed no or little neutralizing capacity whereas the convalescent serum prevented completely the development of cytopathogenic effects.

The use of monkey kidney cells for virus isolations presents certain problems. In the above studies, 11 batches of kidney cells were employed. In cultures of one of these, large "giant cells" appeared in control tubes after 11 days of incubation similar to those produced by the "foamy agent"(3,4). Prior to the 11th day, tubes inoculated with saliva from a mumps patient had shown lesions which were attributed to the presence of mumps virus. Since the cytopathogenic effects induced by these 2 agents thus far proved to be indistinguishable it was essential to determine whether the 2 viruses could be separated by subinoculation of HeLa cells. Cytolysis was observed on transfer of medium from the monkey kidney cultures inoculated with saliva, but not when the medium from the control cultures containing the "foamy agent" was used. Furthermore, neutralization tests identified the agent which was transferred to HeLa cells as mumps virus whereas the foamy agent, which could be maintained only in kidney cells, was not neutralized.

Discussion. Isolation of mumps virus was achieved most readily in the past by inoculation of appropriate specimens into chick embryos by the amniotic route and, after a suitable incubation period (5 to 6 days) the presence of virus was established most conveniently by the demonstration of specific hemagglutinins in the amniotic fluids(5,6.) Strains thus obtained could be maintained initially only by further amniotic passages, but, with increasing adaptation to the new host, they could be passed also serially in the allantois. With such increasing adaptation to the chick embryo the virus apparently loses its capacity to propagate in human cells (strain HeLa) or in monkey kidney epithelium, although it may still exert striking cytolytic effects as long as sufficient quantities are added to the cultures (1). As shown in the present report, strains of virus less extensively adapted to the chick embryo, which had undergone only a few amniotic passages, retain their capacity to multiply in HeLa or monkey kidney cultures and thus it was possible to maintain them for at least 20 transfers in these cells. The lesions observed in these passage series were indistinguishable from those induced by allantoically adapted virus as reported previously(1). The interpretation of the differences in behavior of amniotic and allantoic strains in terms of degree of chick embryo adaptation is further supported by the results of primary isolations of mumps virus in HeLa or monkey kidney cells. Indeed, the tissue culture method for virus isolation appears to be superior to the chick embryo technic both with respect to efficiency and speed. Whereas typical lesions developed directly in the cultures on inoculation of saliva from mumps cases frequently within 3 days, several passages were often required in the chick embryo before hemagglutinins became detectable as the criterion of infection. The HeLa and monkey kidney cells proved to be equally susceptible but cytolysis was more readily discernible and at an earlier time in the former. Aside from this difference, the fact that monkey kidney cells often carry latent viruses renders them less suitable for isolation procedures. The so-called "foamy virus"(3) is particularly disturbing, since the lesions produced by it resemble those caused by mumps virus.

Summary. Strains of mumps virus, which had been isolated in the chick embryo and maintained by up to 6 serial amniotic passages, were readily passed in series in cultures of human cancer cells (strain HeLa) and of monkey kidney epithelium. The lesions observed were dominantly cytolytic in nature. Primary isolations of mumps virus from the saliva of patients with acute parotitis have been achieved in both types of cultures with

an efficiency and speed not matched by the chick embryo technic.

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Kinetics of Human Complement. (21874)

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Considerable work has been done on the separation and identification of the intermediate steps of the reaction between guinea pig complement, (g.p.C′) and sensitized sheep erythrocytes (AE)(1-3). Before undertaking a similar study of the intermediate reactions of human complement (hu C′) and AE, the overall reaction between hu C′ and AE was examined kinetically.

Methods. Sheep blood* in 500 ml lots was preserved with the addition of 2,000,000 units of penicillin "G" and 25 ml of 0.15 M Trisodium ethylenediamine tetraacetic acid(4). Veronal buffer(5) containing 5 x 10^{-4} M Mg^{++} and 1.5 x 10^{-4} M Ca^{++} was used for all dilutions and washings. Pooled human sera, stored in small lots at –25°C, were the source of hu C′. Kinetic analyses were carried out according to the technic of Mayer and Levine(6). Bath temperatures were held constant to ±0.1°C.

Results. 5.0 ml of varying dilutions of hu pool 1 were set up with 10.0 ml of AE and 10.0 ml of buffer. The reaction curves are plotted in Fig. 1. In contrast to the curves obtained with g.p.C′, which are sigmoid(7), these curves are concave to the time axis until the reaction stops. Furthermore, the fact that this reaction is about 90% complete in 10 minutes, even at low C′ concentrations, means that many of the intermediate steps must proceed too rapidly for convenient study. In an effort to obtain reaction times more amenable to study, Exp. 1 was repeated at 32°C, 27°C and 22°C. In Fig. 2, the curves for a single dilution, 1/40, are plotted.

Discussion. With a decrease in reaction temperature two effects are apparent. First, the kinetic curve approaches a sigmoid, second, both the extent of reaction and the time

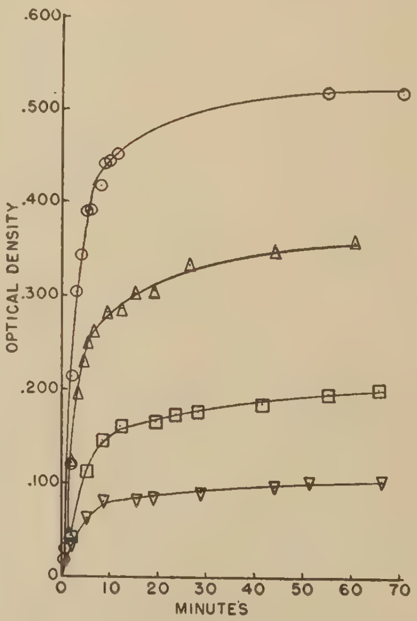

FIG. 1. Lysis of AE by varying dilutions of hu C′; circle, 1/25; pyramid, 1/30; square, 1/40; inverted pyramid, 1/50.

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* The sheep cells were obtained through the courtesy of Dr. L. Pillemer.

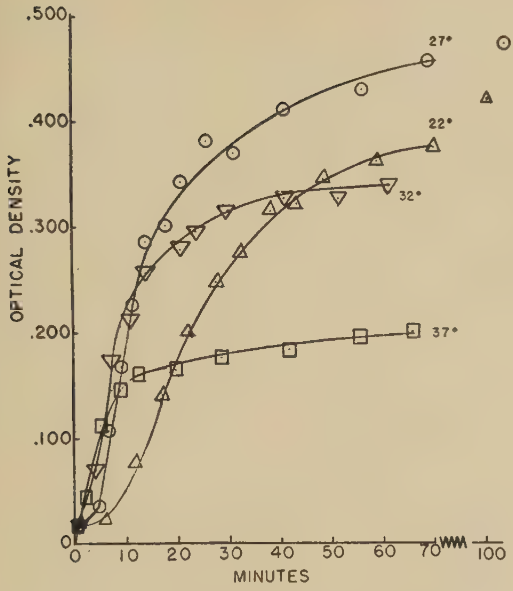

FIG. 2. Lysis of AE by a constant volume of hu C′ 1/40 at varying temperatures.

span for significant reaction greatly increase. Both of these observations can be explained, assuming that the rate of formation and the rate of destruction of intermediate complexes are directly proportional to temperature. These intermediate complexes are currently under study. From purely practical considerations, lytic reactions involving hu C′ might best be carried out at a temperature of 30°-32°C which represents a compromise between optimal lysis and minimal reaction time.

Summary. The optimal temperature for lysis using human C′ is from 30°-32°C. As the temperature decreases from 37°C to 22°C the kinetic curve approaches the sigmoid shape typical of guinea pig C′.

The author wishes to express his appreciation for the technical assistance of Miss Edna Chapman.

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Phagocytosis Augmenting Factor Produced by Leucocytes. (21875)

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During direct study of phagocytosis by phase microscopy and cinematography(1) it was often observed that leucocytes attracted to bacteria made repeated contacts with them before actual ingestion took place. In preliminary studies, brucellae subjected to agitation with washed buffy coat from dog blood were immediately and very actively phagocytosed when subsequently mixed with whole dog blood and observed by phase microscopy. This contrasted strikingly with the delayed and less active phagocytosis of untreated brucellae. Parallel quantitation determinations showed much more phagocytosis of the treated bacteria. Therefore, quantitative experiments were designed to test whether precontact between blood cells and bacteria enhanced phagocytosis of the latter by leucocytes. Agitating bacteria with washed dog blood cells suspended in Krebs-gelatin solution, then separating the bacteria by centrifugation and suspending them in whole blood, accelerated and increased phagocytosis of the organisms while diminishing the frequency of

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TABLE I. Effect of Blood Cell Substance Enhancing Phagocytosis. Mean percentage of neutrophils containing bacteria.

			Bacterial treatment with—						
			Washed blood cells		Supernatant of washed blood cells				
Exp.	No. obs.	Phagocytosis* in	Dog	Guinea pig	Dog	Guinea pig	No treatment	Mean diff.	Significance‡
1	12	Whole dog blood	82				62	20	+++
2	27	*Idem*			60		42	18	++
3	12	"			58		51	7	++
4†	16	"	50		42		27	15	++
5	58	"		39			34	5	+
6	11	Washed dog blood cells	10				11	–1	NS
7	11	Low opsonin human blood	29				26	3	±
8	18	Whole dog blood	71(2×)§				71	0	NS
9	13	*Idem*			76(2×)		76	0	NS
10	17	"				77	78	–1	NS
11	24	Whole guinea pig blood			24		16	8	+++

* At 37°C in Exp. 3; at 25°C in all other experiments.

† In this experiment sensitization by washed blood cells was compared with sensitization by supernatant of washed blood cells and both were compared with no bacterial treatment. While both methods of treatment enhanced phagocytosis, there was no significant difference between the two. The probability figure is therefore given for the supernatant treated versus untreated values.

‡ The probability that the mean difference occurred by a chance of less than 1 in 1000 is indicated by +++, of less than 1 in 100 by ++, and of less than 5 in 100 by +. Borderline significance is indicated by ±, and no significance by NS.

§ (2×) means washed 2 times.

or eliminating pre-ingestion contacts. The following studies demonstrated the novel finding that blood leucocytes produce a substance which enhances phagocytosis demonstrable only when tested in plasma. This sensitizing substance is transferred to bacteria by diffusion from or contact with leucocytes and differs from opsonin.

Methods. Venous blood of dog, man or guinea pig was heparinized with 1 mg of heparin (Lederle) per 10 ml. The blood cells were washed 6 times, each time with 10 volumes of Krebs-gelatin solution to remove opsonin(2). A virulent strain of *Brucella suis* was grown for 24 hours on tryptose agar and suspended in 0.9% NaCl to 1 x 10^{10}/ml, killed by treatment with 1% CH_2O for 24 hours at room temperature, washed 3 times in saline, brought to the concentration cited above and cultured for viability. This served as the stock suspension. In each experiment bacteria from the stock suspension were washed once with Krebs-gelatin solution and resuspended to original concentration in fresh Krebs-gelatin solution. The bacteria were sensitized by agitating equal parts of Krebs-gelatin bacterial suspension and washed blood cells in a rotator revolving 30 times per minute at 37°C. After separation from the blood cells by centrifugation at 700 rpm for 5 minutes, 0.1 ml of the bacterial supernatant suspension was mixed with an equal volume of whole blood at 37°C in Experiment 3 of Table I and at 25°C in all other experiments. Untreated bacterial suspensions similarly mixed with blood served as controls. Smears were prepared at intervals of 7, 15 and 30 minutes, stained with Jenner-Giemsa, and the percentages of 50 neutrophils containing bacteria were tallied. The mean percentages of neutrophils containing bacteria in the 3 observations during the half hour period were recorded. Sensitizing activity of supernatants of washed blood cells agitated without bacteria was also studied. Bacteria were sedimented from supernatant mixtures by centrifugation at 3500 rpm for 30 minutes.

Results. Table I summarizes the experimental procedures, the mean percentages of neutrophils containing treated or untreated bacteria and statistical analysis of the differences. *Agitation with blood cells enhances phagocytosis of bacteria.* Bacteria rotated with washed dog blood cells, separated, and then mixed with whole dog blood were phagocytized more than untreated bacteria, Exp. 1 and 4. *Release from blood cells of substance enhancing phagocytosis of bacteria.* Supernatants of washed dog blood cells agitated in equal volumes of Krebs-gelatin solution for 30 minutes contained a substance which enhanced phagocytosis in whole dog blood, Exp. 2, 3, and 4. The sensitizing effect was not significantly different from that of direct contact with the blood cells, Exp. 4. In contrast to dog, the washed blood cells of guinea pig did not regularly sensitize *Brucella* through contact, Exp. 5, or through the supernatant liquid, Exp. 10. Of 58 observations in Exp. 5, 21 showed either no effect or less phagocytosis, yet the slight difference due to contact approached conventional significance. Although slight hemolysis of dog but not of guinea pig blood cells occurred during the period of agitation for releasing sensitizing substance, 14 sets of observations with washed dog and guinea pig blood cells hemolyzed by distilled water showed no preformed sensitizing substance in them. Thus the sensitizing substance was produced by the dog blood cells during agitation.

Blood cell substance sensitizing bacteria to phagocytosis differs from opsonin. That the sensitizing substance was not opsonin was demonstrated in several ways. Opsonin had been removed by washing the treatment blood cells 6 times with 10-fold volumes of Krebs-gelatin solution(2). There was no evidence that opsonin diffused from washed blood cells when untreated bacteria or bacteria sensitized by previous agitation with washed blood cells were tested with blood cells free of plasma, Exp. 6. Washed blood cells from normal guinea pigs free of blood or tissue opsonin(3) failed to sensitize bacteria regularly, Exp. 5. Washing the organisms twice after their sensitization by washed dog blood cells, Exp. 8, or by the supernatant of washed dog blood cells, Exp. 9, removed the sensitizer, showing that the sensitizer, unlike opsonin, was not fixed on the bacteria.

Mechanism of bacterial sensitization by blood cell factor. The sensitizer did not enhance leucocytic function directly. When plasma was excluded, the washed dog blood cells showed no response to the sensitizer, Exp. 6. Guinea pig whole blood free of opsonin showed greater phagocytosis of bacteria sensitized by supernatant of washed dog blood cells than of untreated bacteria, Exp. 11. Likewise, human whole blood low in opsonin phagocytized bacteria treated by washed dog blood cells more readily than untreated organisms, Exp. 7, although the sensitizing effect just bordered statistical significance. Thus plasma but not opsonin was essential for action of the sensitizer. *Bacterial sensitizing factor produced by white blood cells.* Bacteria were sensitized by treatment with Krebs-gelatin solution containing 7,800 to 15,000 dog blood leucocytes and 70,000 to 140,000 red blood cells per cmm, but not by suspensions containing 40 to 150 leucocytes and 2,250,000 to 3,600,000 red blood cells per cmm. Leucocytes were separated by a method to be described(4). In 14 sets of observations with washed dog blood cells the mean percentages of neutrophils of whole blood phagocytosing bacteria were 48 with bacteria treated with red blood cells, 41 with untreated bacteria and 75 with bacteria treated with white blood cells. The least significant difference was 11. In contrast, 33 similar trials with washed guinea pig blood cells showed values of 21, 28 and 25 respectively, with a least significant difference of 10. Thus, sensitization was produced only by the leucocytic fraction of dog blood but not of guinea pig blood.

Discussion. In the procedure described under Methods, some treated bacteria were unavoidably lost during the centrifugation necessary to separating the bacteria from blood cells. Thus the untreated bacteria were more concentrated than the treated bacteria in the various tests. Since the percentage of neutrophils containing bacteria is related to the bacterial concentration, these conditions weighted the results in favor of greater phagocytosis in the control tests. In preparing the whole blood-bacterial preparations and drawing samples for determining phagocytosis after various intervals, simultaneous manipulation of the experimental and control samples was not possible. A deliberate choice of longer contact in the control tubes also favored greater phagocytosis of untreated bacteria. In spite of these considerations, the data clearly demonstrated greater phagocytosis of treated bacteria under special conditions.

Dog blood leucocytes elaborated a substance sensitizing *Brucella* for phagocytosis, whereas guinea pig blood cells failed to sensitize even through direct contact with bacteria. In contrast, mechanical contact between dog red blood cells had no effect on bacterial sensitization. Dog leucocyte sensitizer is a diffusible substance which was demonstrable with human or guinea pig whole blood. Differences between opsonin and the leucocyte factor which augments phagocytosis have been cited above. Electrokinetic potential and surface charge have been discussed at great length(5) as possible factors influencing phagocytosis. Although sensitization of bacteria by dog blood leucocytes may also occur through a change in surface charge of the bacteria induced by contact with leucocytes, the present experiments indicate no need for direct contact. These experiments not only delineate an hitherto undescribed phenomenon in phagocytosis but also a more active system for preparing brucellae for phagocytosis in the naturally resistant dog than in the guinea pig which is highly susceptible to brucellosis.

Summary and conclusions. A factor sensitizing bacteria for phagocytosis is produced by dog blood cells. Sensitization results from direct contact of *B. suis* with dog but not guinea pig blood cells, or with a product of dog blood leucocytes diffusing into the suspension medium. The sensitizer differs from opsonin in that it is liberated from blood cells washed free of opsonin, from blood cells of animals free of specific opsonins, and is not fixed to sensitized bacteria. The sensitizer requires plasma but not opsonin for its action. It is not found in lysed washed dog or guinea pig blood cells, but is produced in demonstrable quantity by gentle agitation of dog leucocytes for 30 minutes at 37°C; it is not produced by dog red blood cells or guinea pig red or white blood cells.

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In vivo and *In vitro* Studies of Labeled Antibodies Against Rat Kidney and Walker Carcinoma.* (21876)

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The coupling of a radioactive isotope to an antibody, which after systemic administration will localize preferentially in a tissue or organ that it is desirable to radiate, may prove to be a useful means of localizing radioactivity for therapeutic purposes. Such an antibody might be one that would bind specifically with some natural component of the tissue acting as an antigen, or perhaps with an antigenic component of a virus or bacteria preferentially abundant in the tissue in a location to which the antibody could gain access. The object of irradiation will usually be malignant tumor tissue. However, investigations of the specificity of antibody localization that can be obtained in normal tissues may be useful in illustrating principles leading to therapeutical localization of radioactivity.

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Using rat kidney homogenate as an antigen, antibodies have been prepared in rabbits, labeled with I^{131}, and injected intravenously into rats. These labeled antibodies localize strongly in the kidney(1). By radioautographic technics it is known that with similar anti-mouse kidney preparations localization in mouse kidney is largely in the glomeruli (2). Antibodies prepared against other normal rat organs show some tendency to localize preferentially in the homologous organ after intravenous injection(3-5). However, in many instances other organs or tissues approach or exceed the target organ in I^{131} concentration. For example, rat anti-kidney antibodies localize in rat adrenal and ovary in higher concentrations than the average for kidney tissue(5).

Pressman and Korngold have reported that I^{131} labeled antibodies prepared similarly against the Murphy lymphosarcoma of the rat and the Wagner osteogenic sarcoma of the mouse showed some tendency to localize preferentially in these respective tumors(6,7). However, the ratio of resulting radioactivity between tumor and normal tissue seems too low for a significant therapeutic effect. The following experiments were therefore carried out to help advance the therapeutic usefulness of antibodies as carriers of radioactivity. The experimental findings have been reported in part(8).

Experimental. Rabbits were immunized against rat kidney tissue and the gamma globulin from such rabbit serum labeled with I^{131}, using methods previously described(5). The rat kidney homogenate was mixed with the adjuvants used by Salk(9) and injected intramuscularly twice a week for 4 weeks. After iodination a further partial separation of I^{131} labeled antibody from non-antibody material was made by absorbing the iodinated material onto rat kidney homogenate, and later eluting the combined antibody from the washed kidney homogenate by incubation at 60°C, pH 8 for 15 minutes, using general methods previously described(10). This eluted material, designated "purified" anti-kidney antibody, was used in the *in vitro* experiments and for *in vivo* injection. In an exactly parallel manner antibodies were prepared in rabbits against the Walker rat carcinoma 256 and "purified" by absorption and subsequent elution from tumor tissue. Analogous control preparations were made using the serum from non-immunized rabbits.

In the *in vivo* experiments rats were sacrificed by perfusion with 750 ml saline to render tissues largely blood-free. Their drinking water contained 0.001% inorganic non-radioactive KI for several days before the labeled antibody injection. In the *in vitro* experiments portions of the "purified" labeled antibodies were incubated in 10 ml cellulose nitrate tubes with various concentrations of tissue homogenates as antigen, 0.5 ml normal rabbit serum as a protective protein, and borate buffer pH 8 to a total volume of 3 ml. Incubation was for 1 hour at 37°C in a Dubnoff shaking incubator. Tubes were then centrifuged, the supernatants discarded or transferred to other tubes for additional incubations, and radioactivity of the tissue residues measured before and after washing with 3 ml portions of borate buffer. All radioactivity measurements were made by gamma ray measurements, using a well-type scintillation counter.

Results. Fig. 1 shows the results of an experiment in which a "purified" I^{131} labeled rat kidney antibody preparation was incubated with varying amounts of rat and rabbit kidney homogenate. The results suggest that at least 25% of the I^{131} present is combined with antibody that reacts with, and is bound specifically to, rat kidney as compared with

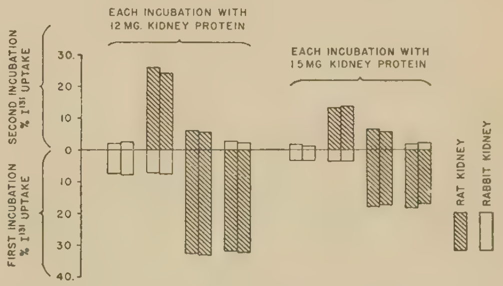

FIG. 1. Per cent I^{131} uptake by washed kidney residues in duplicate experiments in which rat or rabbit kidney homogenates were incubated with an I^{131} labeled rabbit gamma globulin fraction containing antibodies against rat kidney. The supernatant, after first incubation for 1 hr, was removed and used for second incubation.

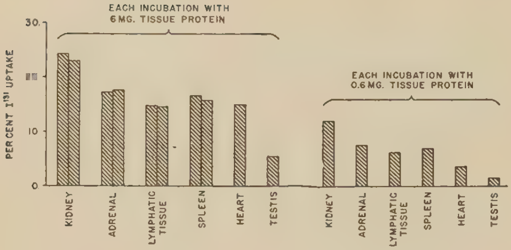

FIG. 2. Per cent I^{131} uptake by washed rat organ residues in single and duplicate experiments in which rat organ homogenates were incubated with an I^{131} labeled rabbit gamma globulin fraction containing antibodies against rat kidney.

rabbit kidney. Also, a preliminary incubation with rabbit kidney does not destroy the affinity of the labeled protein for rat kidney on a subsequent incubation.

Auxiliary experiments showed that the preferential binding of I^{131} to rat kidney tissue is almost completely suppressed if before incubation with the labeled material the kidney homogenate is first incubated with 1 ml of unlabeled rabbit anti-rat kidney serum. Presumably most potential antibody sites are occupied by unlabeled antibody materials during this first incubation and are not available for binding of labeled antibody.

Fig. 2 shows the results of a similar experiment in which this labeled anti-rat kidney preparation was incubated with homogenates of various rat organs. Greater binding is shown for kidney than other tissues, but the degree of binding by all rat tissues is significantly greater than the rabbit kidney binding shown in Fig. 1.

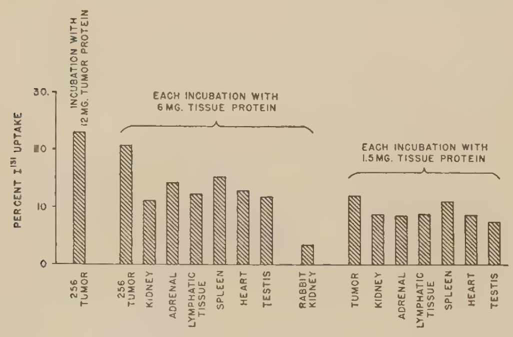

FIG. 3. Per cent I^{131} uptake by washed rat organ residues and a rabbit kidney residue in experiments in which these organ homogenates were incubated with an I^{131} labeled rabbit gamma globulin fraction containing antibodies against the Walker rat carcinoma 256.

Fig. 3 shows the I^{131} binding that resulted when the "purified" labeled anti-Walker tumor antibody preparation was incubated with various rat organs, rat tumor tissue, and with rabbit kidney. In this case, *in vitro* tumor specificity is shown by greater I^{131} uptake by tumor than other tissues, but binding to all rat organs studied was substantially greater than the rabbit kidney values.

Fig. 4 shows the distribution of I^{131} resulting when the same "purified" anti-kidney preparation used in the *in vitro* experiments was injected intravenously into rats. These results are consistent with those of many similar experiments. The material is kidney specific in the sense that localization in the kidney is far higher than for the control material. But there is also substantial localization in spleen, and on a concentration basis localization in adrenal and ovary is often higher than in the kidney itself.

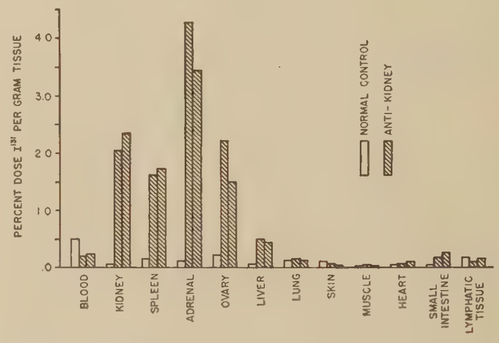

FIG. 4. Distribution of I^{131} in 2 rats 3 days after intravenous injection of an I^{131} labeled rabbit gamma globulin fraction containing antibodies against rat kidney. Control values were obtained after injection of a similarly prepared fraction of normal rabbit gamma globulin. Data normalized to 300 g animals.

Fig. 5 shows the distribution of I^{131} resulting from intravenous injection of the anti-Walker tumor labeled antibody preparation. In the rats receiving the anti-tumor material the highest I^{131} concentrations are reached not in tumor, but *rather* in adrenal and ovary. These general results are confirmed by other similar experiments.

Fig. 6 shows the distribution of I^{131} follow-

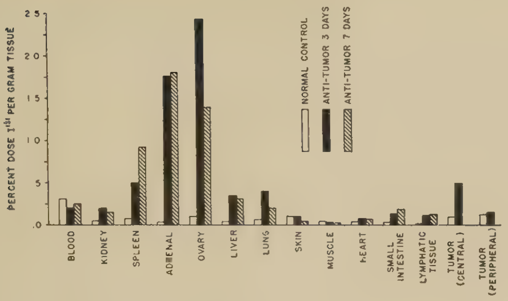

FIG. 5. Distribution of I^{131} in rats after injection of an I^{131} labeled rabbit gamma globulin fraction containing antibodies against the Walker rat carcinoma 256 or a similarly labeled fraction of normal rabbit gamma globulin. The 2 animals perfused at 3 days after injection carried the Walker tumor; the 7 day animal did not. Data normalized to 300 g animals.

ing injection, by intravenous, intraperitoneal, and intramuscular routes, of another similar I^{131} labeled "purified" anti-kidney antibody. Very little of the kidney specific antibody, as determined by I^{131} abundance, appears in the kidney following the intramuscular route of administration, although I^{131} blood values are similar by the 3 routes of administration, suggesting that the antibodies that would have localized in kidney after intravenous injection were bound near the injection site following intramuscular injection and that the material reaching the blood stream was mostly iodinated non-specific protein material. In a comparable experiment labeled anti-tumor antibodies, injected subcutaneously, did not localize in adrenal or ovary.

Fig. 7† shows the distribution of I^{131} following the injection of labeled "purified" Walker tumor antibodies directly into tumor.

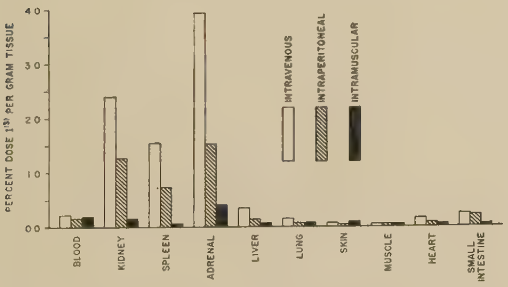

FIG. 6. Distribution of I^{131} in organs of perfused rats 3 days after injection by 3 routes of an I^{131} labeled rabbit gamma globulin fraction containing antibodies against rat kidney. Data normalized to 300 g animals.

The I^{131} localization in adrenal, ovary, and spleen is much reduced compared with localization after intravenous administration of the same substance, and much of the administered radioactivity has remained localized in tumor.

The anti-tumor material injected into tumor was initially in soluble form. If an analogous material were used for infiltrating areas invaded by cancer in human patients, a more uniform tissue concentration might be achieved than with colloidal materials now in use, such as radiogold. Also I^{131} liberated from antibody would be rapidly excreted in a patient given an adequate preliminary dosage of inorganic non-radioactive iodine.

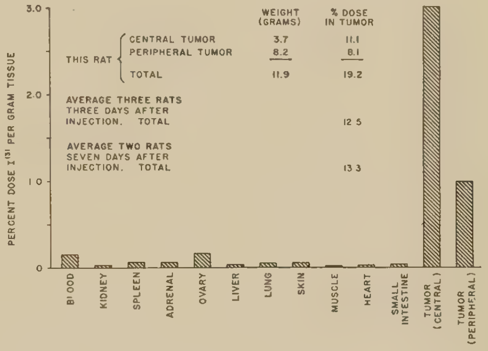

FIG. 7. Distribution of I^{131} in a perfused rat carrying Walker carcinoma 256. Injection of an I^{131} labeled rabbit gamma globulin fraction containing antibodies against this tumor was made directly into the tumor 3 days previously. Data normalized to a 300 g animal. The table shows other data on I^{131} retention in tumors from similar experiments.

The data published here, plus other reported work, suggest the following conclusions. Several rat organs, injected into rabbits, cause the production of antibodies that react *in vitro* with the parent organ homogenate with considerable specificity as compared with other rat organs. However, considerable cross-reaction does occur. Many of these antigens *in vivo* seem to be in locations where circulating antibodies have no, or very limited access to them. This we infer from experimental evidence that anti-Walker tumor antibodies localize in ovary, adrenal, and spleen in much higher concentrations than in tumor tissue, and from other data(11) showing that rat anti-lymph node antibodies localize in spleen in far higher concentrations than in lymph nodes following intravenous injection. This limitation in access could be due to antigen being located inside the cell, or in relatively inaccessible although extracellular spaces, or even to relatively poor circulation for one organ as compared with another, for example, Walker tumor contrasted with adrenal, or lymph nodes with spleen. Finally, all or nearly all antibodies against specific rat organs seem also to be largely generalized anti-rat antibodies in that under some circumstances they can find *in vivo* reaction sites in other rat organs and tissues since only a small portion of anti-rat kidney antibody injected intramuscularly ever reaches kidney and after subcutaneous injection anti-Walker rat tumor antibodies are not present in significant amounts in these organs where they are highly localized after intravenous injection.

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† These data have also been included in a paper submitted to the International Conference on Peaceful Uses of Atomic Energy.

Possible methods of obtaining anti-organ antibodies without this species specificity are being investigated.

Summary. An *in vitro* method is described for demonstrating the binding by rat organ homogenates of I^{131} labeled antibodies produced by rabbits immunized with particular rat organs. By this test anti-kidney antibodies show specificity for kidney compared with other rat tissues, and anti-Walker rat carcinoma 256 antibodies specificity for this tumor tissue. These data are compared with I^{131} distributions found after injection of these antibodies by various routes into living rats. The results suggest that nearly all of these antibody molecules are also general anti-rat antibodies in that they are bound by components of other rat tissues. Such factors as relative circulation rates, blood vessel permeability to proteins, and intracellular or extracellular location of antigens, as well as relative organ specificity of antibodies as demonstrated by *in vitro* experiments, are probably therefore important in determining the localization of such antibodies in animal injection experiments.

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Effect of Cortisone on Lung Metastasis Production by a Transplanted Mammary Adenocarcinoma in Mice.* (21877)

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There are contradictory reports in the literature as whether the repeated administration of cortisone to tumor bearing mice increases the metastatic spread of the tumor. In these experiments the effect of variable doses of cortisone on lung metastasis production by a transplantable adenocarcinoma in mice is reported.

Method. 186 female mice of the ZBC† stock weighing between 16 and 18 g were used. Mice of this stock do not possess the "Mammary Tumor Agent" and are able to grow tumors from mice of the Z (C3H) strain. The tumor used was an adenocarcinoma which arose spontaneously in a Z (C3H) breeder mouse and has been maintained in this laboratory for 52 successive passages into ZBC mice. All mice were inoculated subcutaneously in the right side of the body with 0.2 cc of an 8% cell tumor suspension in saline. Eight days later, when all mice had developed tumors of a mean size of 0.6 cm, the animals were divided in different groups, each one receiving daily a given amount of cortisone acetate diluted in saline. The doses of cortisone were: 4, 12, 37, 111, 333, 1000 and 3000 μg per mouse per day in 0.2 cc volume. The controls received only saline. Mice were injected subcutaneously during 10 days and 7 days later all animals were sacrificed, the primary tumors dissected out and weighed, and the incidence of lung metastases determined by the presence of macroscopic nodules on the surface of the lungs under dissecting scope (1.3 X 10 magnification). The character of the nodules was determined by histological study.

Results. The results obtained are shown in Table I. The incidence of lung metastases in the saline-treated controls was 37.5%. All the cortisone-treated groups, regardless of the dose injected, showed reduction in metastasis incidence, the 3000 μg group having the lowest (11.1%). Only the groups receiving 333, 1000 and 3000 μg showed a significant retardation of the primary tumor growth as compared with the saline controls.

The effect on the growth of the animal (without the tumor) as well as the mortality observed during and after the treatment are seen in the table.

In view of these findings, particularly on the inhibition of lung metastases, it was decided to repeat the experiment using the dose of 1000 μg under the same conditions as before. The results obtained are shown in Table II. Essentially the same results were recorded as far as the incidence of lung metastases, action on the growth of the primary tumor and on the growth of the animal were concerned.

Discussion. It seems clear, therefore, that by using this transplantable adenocarcinoma and under the experimental conditions described, cortisone inhibited the metastatic spread of the tumor to the lungs. Doses as low as 4, 12, and 37 μg were used without affecting either the growth of the animal or that of the tumor and produced a definite decrease in lung metastases. Similarly, doses of cortisone which produced a marked effect on the growth of the animal, as well as on that of the primary tumor, also inhibited metastasis. In this respect the 3000 μg dose was particularly effective.

Our results do not agree with those reported by Agosin *et al.*(1), Molomut *et al.*(4) and Baserga *et al.*(2), who found that cortisone administration to tumor bearing mice in-

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* Aided by grants from the Minnesota Division of the American Cancer Society and an institutional grant from the American Cancer Society.

† ZBC mice are obtained as follows:

Ax(A without the MTA) X Zb (Z without the MTA)

↓

AxZb F_1 X Zb

↓

ZBC

TABLE I. Effect of Repeated Administration, during 10 Days, of Several Doses of Cortisone Acetate on Incidence of Lung Metastases in Mice Bearing a Transplanted Adenocarcinoma.

Cortisone, μg/mouse /day	No. of mice	Initial body wt, g (mean)	Final body wt, g (mean)	Changes in body wt, %	Mortality		Final tumor wt, g (mean)	Incidence of lung metastases
					During treat.	After treat.		%
None	24	18.33	18.88	+ 3.0	0	0	4.83 ± .5	9/24 (37.5)
4	24	17.95	18.95	+ 5.5	1	0	5.54 ± .5	4/23 (17.3)
12	24	16.66	17.61	+ 5.6	0	0	3.70 ± .2	4/24 (16.6)
37	24	16.82	17.23	+ 2.4	1	1	5.60 ± .4	6/23 (26.0)
111	23	16.92	17.18	+ 1.5	0	0	5.74 ± .4	5/23 (21.7)
333	23	17.77	18.00	+ 1.2	0	0	3.26 ± .3	4/23 (17.3)
1000	24	17.04	16.00	− 6.1	2	2	1.55 ± .2	5/22 (22.2)
3000	24	16.22	13.70	−15.5	6	6	1.08 ± .2	2/18 (11.1)

TABLE II. Effect of Repeated Administration, during 10 Days, of Cortisone Acetate on Incidence of Lung Metastases in Mice Bearing a Transplanted Mammary Adenocarcinoma.

Group	Cortisone, μg/mouse/day	No. of mice	Final body wt, g (mean)	Final tumor wt, g (mean)	Incidence of lung metastases
Controls	None	17	22.26	5.17	5/17 (28.2%)
Treated	1000	26	20.40	1.31	2/26 (7.6%)

creases the metastatic spread of the tumor. However they are more in line with the results obtained by Kaliss *et al.*(3), who by using the same experimental conditions as those used by Molomut *et al.*, did not find more metastases in the treated than in the untreated mice. Part of the discrepancy between our results and those reported by Agosin *et al.*, and Baserga *et al.*, might be due to the fact that they administered cortisone for a longer period than 10 days as it was used in our experiments. That would indicate, perhaps, that cortisone might have an inhibiting action when administered for short periods and a subsequent enhancing effect when administered for 30 or more days as reported by Agosin *et al.* Since there is also the possibility that not all mammary adenocarcinomas will respond in the same fashion to cortisone treatment, perhaps the discrepancy in the results might be due to the type of tumor used in the experiment. In fact the Chilean workers have used a transplantable adenocarcinoma which does not metastasize spontaneously, whereas the tumor used in these experiments, also a transplantable adenocarcinoma, does metastasize spontaneously to the lungs in most of the animals when growing in the subcutaneous tissue.

At any rate, it can be said at this time, that under the experimental conditions used in the present experiments, repeated administration of variable doses of cortisone even in amounts as low as 4 μg per day, per mouse, during 10 days, reduced rather than increased the metastatic spread of the tumor to the lungs as compared to the untreated controls.

Summary. Repeated administration of cortisone acetate in doses ranging from 4 to 3000 μg, injected during 10 days into mice bearing a transplantable mammary adenocarcinoma, inhibited the spontaneous production of lung metastases.

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Chemotherapeutic Activity of Some Hydroxysulfapyridines. (21878)

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When sulfapyridine is fed, it is converted in part to 5′hydroxysulfapyridine, and this in turn is transformed, presumably in the liver, to a water-soluble hydroxysulfapyridine glucuronide(1,2). In the course of study of the urinary excretion of sulfapyridine we had occasion to synthesize 5′hydroxysulfapyridine and its 3′ and 6′ isomers(3). Since the glucuronide does not enter into urolith formation, it seemed desirable to test its precursor, the hydroxysulfapyridine, in infected animals to determine whether or not it would afford protection without the hazard of urolithiasis.

Experiments in vitro. The inoculum consisted of 0.1 ml of a 1 x 10^{-4} dilution of a 6-hour culture of *Streptococcus pyogenes* (ATCC No. 8668) grown on peptone dextrose broth at 37° according to the method described by White(4). The inoculum, containing 4000 cells as determined by plate count, was added to 10 ml of broth containing the appropriate hydroxysulfapyridine or a standard drug in a concentration of 400 p.p.m. Following a serial 2-fold dilution the tubes, incubated at 39°, were examined after 48 hours and again at a later interval.

The same procedure was followed using strains of *E. coli* (ATCC No. 26 and ATCC No. 11303) except that the medium was that reported by Herriot and Barlow(5), the inoculum contained 6000 cells, and the incubation was carried out at 37°. The results of both experiments are given in Table I.

Experiments in vivo. Two series of tests were performed following Streptococcus infection and a third series of tests involved infection with *E. coli. Streptococcus pyogenes* (ATCC No. 8668) was grown in a heart infusion broth (Difco) containing 5% defibrinated rabbit blood. Virulence was maintained by frequent mouse passage. The culture was diluted 1 x 10^{-4} and 0.5 ml of this culture (containing approximately 5000 cells) was injected intraperitoneally. *E. coli* (ATCC No. 26) was grown in a peptone dextrose broth and the culture was diluted to 1 x 10^{-4}. A final dilution to 1 x 10^{-5} was made with 5% gastric mucin. Of this, 0.5 ml, containing approximately 5,000 cells was given intraperitoneally. In our hands, ATCC No. 8677, 11303, Brown University Standard No. 261, as well as 3 laboratory strains of *E. coli* were not lethal to mice.

Standard drugs and test compounds were pulverized and suspended in concentrations of 2% in water containing 10% gum acacia. In the first test with *S. pyogenes,* each mouse received 0.5 ml of suspension containing 10 mg of drug 5 to 15 minutes before intraperitoneal infection and then once daily for 5 additional days. In the second test only 5 mg of drug was given before infection and this dose was then continued twice daily. In the third test with *E. coli* the 10 mg of drug was given at one time as in the initial experiment. Animals were observed for a period of 10 days. Groups of 15 male albino CF-1 mice weighing 19 to 25 g were used in each experiment with 5 animals used as controls. In some instances duplicate or triplicate series were run, but in the interests of space the data are combined in Table II.

Results and discussion. 5′-Hydroxysulfapyridine, the metabolite which is excreted in the urine following oral administration of sulfapyridine, appears to be as active *in vitro* as the parent drug. The isomeric 3′ and 6′-hydroxysulfapyridines appear to be as active as sulfapyridine against *S. pyogenes* but not as active against *E. coli* (Table I).

In spite of the *in vitro* activity of the hydroxysulfapyridines, their activity *in vivo* is

TABLE I. Antibacterial Activity of Hydroxysulfapyridines *in vitro.* 48 hr readings. Concentrations are given in p.p.m.

Compound	*S. pyogenes*	*E. coli* (#11303)	*E. coli* (#26)
3′-Hydroxysulfapyridine	25	6.3	3.1
5′- ”	50	1.5	1.5
6′- ”	50	25.	25.
Sulfapyridine	50	1.5	3.1

TABLE II. Chemotherapeutic Activity of 10 mg Oral Doses of Various Sulfonamides Given for 6 Days after Intraperitoneal Infection in Mice. In Exp. 2, the drug was given twice daily in 5 mg doses.

No. of mice	Compound	Dilution of culture	% survivals in days 1	2	3	4	5	6	7	8	9	10
	Exp. 1. Organism, *S. pyogenes*											
15	3'-Hydroxysulfapyridine	10^{-4}	0									
15	5'- "	10^{-4}	80	13	13	13	13	13	0			
15	6'- "	10^{-4}	20	0								
45	Sulfapyridine	10^{-4}	100	96	96	93	93	93	82	47	36	22
15	Sulfanilamide	10^{-4}	100	100	93	93	93	93	67	40	20	13
30	Sulfisoxazole	10^{-4}	97	83	77	63	53	43	20	20	17	10
	Exp. 2. Organism, *S. pyogenes*											
15	5'-Hydroxysulfapyridine	10^{-4}	53	20	7	7	7	7	7	7	7	7
15	Sulfapyridine	10^{-4}	100	100	100	100	100	100	80	67	47	27
25	Controls	10^{-4}	4	0								
20	"	10^{-5}	5	0								
20	"	10^{-6}	20	5	0							
25	"	10^{-7}	60	24	12	8	8	8	8	4	4	4
25	"	10^{-8}	100	60	60	60	56	56	56	56	56	56
	Exp. 3. Organism, *E. coli*											
15	5'-Hydroxysulfapyridine	10^{-5}	27	0								
15	Sulfapyridine	10^{-5}	100	100	100	100	100	100	100	100	100	100
10	Controls	10^{-5}	0									
5	"	10^{-6}	0									
5	"	10^{-7}	40	20	20	20	20	20	20	20	20	20

negligible. It may be noted (Table II) that while sulfanilamide and sulfapyridine afford excellent protection against streptococcal infection, the protection diminishes after administration of the drugs is discontinued. Under the conditions of these experiments(6), sulfisoxazole does not appear to be as effective as sulfapyridine. The 5'-hydroxysulfapyridine seems, initially, to afford some protection, but its activity is dissipated within 48 hours. Giving the drug in divided doses did not materially alter the outcome of the experiment (Table II, Exp. 2).

It seems reasonable to assume that the bodily processes which convert the hydroxysulfapyridines to water-soluble excretion products may be the very processes which bring about their loss of activity *in vivo*. When urine from animals receiving 5'-hydroxy-sulfapyridine was examined by means of a distribution technic(7), it was found that 100% of the hydroxysulfapyridine was converted to an unidentified water-soluble conjugate which is presumed to be largely the glucuronide. *In vitro* antibacterial tests with urine concentrates indicated that this conjugate is much less active than an equivalent weight of the hydroxysulfapyridine. In contrast, those animals receiving sulfapyridine excreted only 65% of the so-called "urinary sulfapyridine" in the form of the relatively inactive hydroxysulfapyridine glucuronide.* For these reasons it is suggested that the hydroxysulfapyridines may lose activity *in vivo* because of conjugation and excessively rapid elimination.

Summary. 5'-Hydroxysulfapyridine, a urinary excretion product of sulfapyridine is as active *in vitro* as sulfapyridine against *S. pyogenes* and *E. coli*. It is without significant protective power in mice infected with these organisms. It is suggested that the loss of activity *in vivo* may be attributed to conjugation and excessively rapid elimination. Similar results were observed with the 3' and 6' isomers.

We are indebted to Henry G. Frank for his help in carrying out the microbiological determinations.

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* These observations raise questions about the interpretation of blood concentration and clearance data reported in the past.

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Rate of Metabolism of Ethyl Alcohol in the Mouse.* (21879)

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The metabolism of ethyl alcohol‡ in the mouse has been studied by Nicloux(1) and Le Breton(2) who injected alcohol and after a certain time analyzed the entire animal for alcohol. More recently radioactive alcohol has been used(3,4). We have investigated the rate of metabolism of alcohol in the mouse by following the decline of the concentration of alcohol in the blood after intraperitoneal injection. Certain data obtained led us to suspect and later confirm that during the first hour after injection alcohol is oxidized at a rate much more rapid than the constant rate during the subsequent hours. This finding would appear to be of some importance in our conception of the metabolism of alcohol.

Methods. The mice were all males, weighing 25 to 35 g, maintained at a room temperature of 28°C. Free access was allowed to Purina Chow, unless fasting experiments were undertaken. The alcohol was given as a 20% (W/V) solution usually in a dose of 4 g§ per kilo intraperitoneally. In certain experiments the dose was decreased to 2 g per kilo; in others the usual dose was given intravenously or by mouth. Blood was drawn either from the tip of the tail or, when the animal was sacrificed, from the heart. In initial experiments, it was found that the concentration of alcohol in tail blood might be lower than that in heart blood when mice were used at a room temperature of 15-18°C. However, when the mice were kept at 28°C and experiments performed at this temperature blood from the tail and from the heart contained the same concentrations of alcohol. Analyses of peritoneal fluid from mice given intraperitoneal injections of alcohol and sacrificed in 1 hour indicated that more than 97% of the alcohol had been absorbed. Mice were prepared for analysis of their total content of alcohol by cutting with scissors and homogenizing in a Waring blender containing water and Folin-Wu tungstate; the mixture was diluted to 500 g with water.

Alcohol was determined by using crystalline alcohol dehydrogenase and diphosphopyridine nucleotide‖ essentially as described by Marshall and Fritz(5). The blood obtained from the tail or heart (0.02 ml) was diluted to 10 ml with precipitation of the proteins by Folin-Wu tungstate and distilled without filtration. Five ml of the distillate were collected in a 5 ml graduated flask and an aliquot (2-4 ml) taken for the alcohol determination. One ml of a 0.5 molar buffer of the composition of that of Bonnichsen and Theorell(6) was used.

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* This investigation has been aided by a grant from the U. S. Public Health Service.

† E. R. Squibb Fellow in Pharmacology and Experimental Therapeutics.

‡ Alcohol will be used subsequently when referring to ethyl alcohol.

§ The alcohol was given by means of a syringe pipette. This was adjusted for the mean weight of the mice used in any experiment. Hence, each mouse did not receive exactly 4 g per kilo.

‖ Some samples of DPN obtained from Sigma Chemical Co. or Pabst appeared to contain alcohol which could be removed by dissolving the DPN in water and lyophilizing.

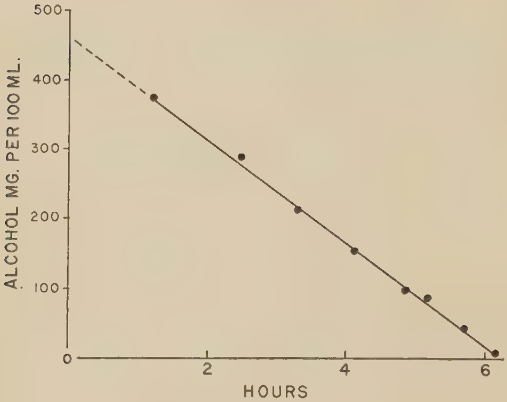

FIG. 1. Concentration of alcohol in tail blood of one mouse determined at various times after intraperitoneal injection of 4000 mg of alcohol per kilo. Straight line fitted to values plotted arithmetically. Slope of line gives 74 mg % per hr as rate of oxidation of alcohol. Extension of line to zero gives 465 mg % as initial concentration of alcohol. Dose divided by initial value gives r of 0.86. 740 × 0.86 gives 640 mg/kg/hr as rate of oxidation.

A portion of the mouse homogenate was centrifuged and an aliquot distilled similarly to blood. In all instances except when tail blood was used duplicate determinations were done. The syringes used for injection were always checked in an experiment by injecting the solution of alcohol used into a suitable volumetric flask nearly filled with water and determining the amount of alcohol delivered. Blank determinations on normal mice treated by the above procedure showed no "alcohol" present. Alcohol added to homogenized mice in amounts similar to those determined indicated that recovery was satisfactory, the maximum error being less than 4%. The rate of metabolism of alcohol has been expressed as mg/kg per hour oxidized. This figure has been calculated from the rate of decline of the concentration in blood and the r (ratio of concentration of alcohol in whole animal to concentration in the blood) or obtained by analysis of the whole mouse.

Results. The concentrations of alcohol in blood when plotted against time have always yielded a straight line as the best fit for the points. This means that the rate of oxidation of alcohol in the mouse is constant and independent of the amount present in the animal when observations are started one hour after administration of the alcohol. A typical result is shown in Fig. 1. The mean rate of oxidation of alcohol in 6 mice was 713 (623-800) mg/kg per hour. This rate of oxidation of alcohol in the mouse is extremely high when calculated on a weight basis; this is not true when the rate is referred to surface area. Thus, taking the mean figure, one can calculate that the rate of oxidation of alcohol in the mouse is 2440 mg per sq. m. per hour. On the basis that man oxidizes 100 mg per kg per hour, his rate would be about 2280 mg per sq. m. per hour. The r varied from 0.86 to 1.00. Alcohol is generally stated to be distributed in total body water, but it is impossible that the amount of body water in the mouse can be 86 to 100% of the body weight. Obviously, either alcohol is distributed in a space greater than that of total body water or the r obtained by projecting the line back to zero time is in error.

If the r calculated by projecting the line back to zero were in error, then the r determined directly by analyzing the whole mouse should not agree with the calculated value. This was tested in a number of experiments; the r obtained directly was always lower than the calculated r. A possible explanation of this was that the rate of oxidation of alcohol during the first hour after administration was greater than in subsequent hours. The fact that the average rate of oxidation determined by analysis of the mouse 3 hours after administration of alcohol was greater than that calculated from the decline in the concentration of alcohol in the blood supported this hypothesis. Experiments were then done comparing the rate of metabolism of alcohol by sacrificing animals ½, 1 and 3 hours after administration of alcohol. The rate of oxidation determined from the ½ and 1 hour points has been much greater than that from the 3 hour point. Also, all of these values have been higher than the rate of oxidation calculated from the rate of decline of blood alcohol.

A summary of the results is given in Table I. The average r calculated from the line was 0.89 while that determined directly was 0.72. The fact that the r determined at ½ hour was the same as the r determined at 1 or 3 hours indicates that absorption was complete in ½ hour. The average calculated rate of

TABLE I. Rate of Oxidation of Alcohol in the Mouse.

	Rate, mg/kg/hr*		r	
Duration of exp., hr	Calculated decline blood alcohol	Direct	Calculated	Direct*
3	532	706	.90	.69
3	573	670	.83	.71
3	459	545	.79	.71
3	636	797	.98	.75
3	572	747	.91	.67
3	365	652	.96	.67
3	613	717	.83	.76
1		1220		.77
1		1240		.73
1		950		.71
1		1080		.73
.5		1370		.68
.5		1540		.75
.5		1040		.70
.5		1400		
.5		1260		

* Each line of table represents an experiment on one mouse.

oxidation was 536 mg per kg per hour; that directly determined was for ½ hour 1322, for 1 hour 1123, and for 3 hours 691 mg per kg per hour. The conclusion appears to be inescapable that in these experiments although the rate of oxidation of alcohol in the mouse is constant and independent of the amount present after 1 hour, the rate of oxidation is much greater during the first hour after administration of alcohol.

It must be clearly understood that the results presented so far have all been obtained on mice given 4 g per kilo of alcohol intraperitoneally and allowed free access to food until the start of the experiment. To define some of the factors responsible for the increased oxidation of alcohol during the first hour, we have carried out experiments to determine the effect of variations in procedure such as dose, route of administration and fasting. A summary of the results is given in Table II. Reducing the dose from 4 to 2 g either abolished or markedly reduced the initial increased rate of oxidation of alcohol. The effect is still observed when the alcohol is given by mouth or intravenously, although in the latter case the increase in the first hour appears to be less. When mice were fasted for 9 hours, the increase rate of oxidation during the first hour was still observed when alcohol was given either intraperitoneally or by mouth.

Discussion. When alcohol is given by intraperitoneal injection to a mouse and the alcohol content of blood determined at various intervals starting 1 hour after injection, the rectilinear relation of the points plotted against time indicates that alcohol disappears at a constant rate independent of the amount present. However, direct determination of the total amount of alcohol disappearing from the entire animal shows that the disappearance is much faster during the first hour than in subsequent hours.

We have assumed that the alcohol which disappears has been oxidized. The amount of alcohol excreted as such in the urine and expired air would be small and could not account for the difference between first and third hours. Casier(4) found in the mouse 93% of intraperitoneally administered alcohol to be excreted as CO_2 in 25 hours; Skipper *et al.*(3) also, found 92% of the administered alcohol eliminated at $C^{14}O_2$ in 24 hours in the mouse. Both of these workers found

TABLE II. Effect of Various Factors on Rate of Oxidation of Alcohol in the Mouse.

Condition	Dose, g/kg	Route	Time, hr	No. mice	Avg mg/kg/hr	Range mg/kg/hr
Non-fasted	2	I.P.*	.5	3	650	500- 800
”	2	I.P.	2.5	3	585	554- 606
”	4	I.V.	1	5	917	754-1120
”	4	I.V.	3	4	760	688- 787
”	4	P.O.	1	2	1245	1200-1290
”	4	P.O.	3	2	919	860- 978
Fasted	4	I.P.	1	3	916	667-1090
”	4	I.P.	3	3	536	450- 648
”	4	P.O.	1	2	1220	1010-1430
”	4	P.O.	3	2	691	690- 692

* I.P., by intraperitoneal inj.; I.V., by intravenous inj.; P.O., by stomach tube.

some C^{14} retained in the mouse at 24 hours. Obviously, the amount excreted in the urine and expired air is negligible. These results of Casier and Skipper *et al.* eliminate the possibility that some alcohol in the first hour is not oxidized but converted to some other compound as claimed in the dog by Widmark (7). Hence, we must believe that oxidation during the first hour is much greater than in subsequent hours.

A study of various factors indicates that the more rapid rate of oxidation during the first hour is dependent on the use of a large dose. Thus, with the 2 g per kilo dose, no significant increase in the rate of oxidation is seen in the first ½ hour as is consistently observed with the 4 g dose. An increase in the first hour certainly occurred when alcohol was given by mouth. Fasting the mice also did not abolish the increase in the first hour whether the alcohol was given intraperitoneally or by mouth. When intravenous injection was used less increase was observed during the first hour than when the alcohol was given intraperitoneally or by mouth.

It appears from the above facts that the increase in the rate of oxidation of alcohol is related in some way to a very high dose. Both intraperitoneal and oral routes would insure a high concentration of alcohol in portal vein blood. Decreasing the dose would reduce this concentration. In the case of intravenous injection, the alcohol was administered in the course of 2 to 6 minutes. A very high concentration would reach the liver before distribution to the tissues. The above facts suggest as one possibility that the increase rate of oxidation during the first hour is related to a high concentration of alcohol reaching the liver.

It is clear that although alcohol is oxidized at a more or less constant rate as shown by the rate of decline in the concentration of alcohol in the blood, under certain circumstances the rate of oxidation can be doubled during the first half-hour after administration of the drug. This means that whatever limiting factors account for the constant rate of oxidation, these can be modified under certain conditions. To what extent this increased rate of the oxidation of alcohol occurs in other species, particularly man, remains to be determined. If in man it occurs under certain conditions when alcohol is given by mouth, it would appear to make the estimate of the maximal rate of oxidation entirely too low.

Summary. The rate of oxidation of alcohol has been studied in the mouse by determination of the rate of decline of the concentration of alcohol in the blood as well as by determination of the alcohol content of the whole animal at various times after administration of the drug. When observations are begun one hour after administration of alcohol, the rate of oxidation is constant and independent of the amount present. However, the rate of oxidation may be twice as great in the first hour as in subsequent hours. The effect is dependent on dose and to some extent on mode of administration.

We wish to thank Sara Ann Verplanck for technical assistance.

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Clearance of Bacteria from the Blood of Irradiated Rabbits. (21880)

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Recent studies on the pathogenesis of post-irradiation infection have directed attention to the activity of the reticulo-endothelial system, particularly its hepatic and splenic components, in removing bacteria from the blood stream of the irradiated mouse(1,2).

The effect of irradiation on the ability of the reticulo-endothelial system to remove inanimate particulate matter from the circulating blood has been studied by several groups of investigators using radioactive $CrP^{32}O_4$, ThO_2 and Au^{198}. Gabrielli and Auskaps(3) found no change in rats after 100 r or less, as did Esplin, *et al.*(4) in mice exposed to 350 r, and Barrow, *et al.*(5) in rabbits exposed to 800 r. Taplin, *et al.*(6), however, in their studies on the clearance rate of prodigiosin from the blood of rabbits, found that although 300 r had little effect, 800 r or more resulted in a depression of the phagocytic function of the macrophage system for a period of 2 weeks. Callaway and Kerby(7) found that splanchnic removal of *Staphylococcus aureus* was not significantly reduced in rabbits after 800 r total body irradiation.

Methods. Healthy young adult rabbits of both sexes,* weighing approximately 2 kg were used. *X-irradiation* was delivered at 250 kv, 30 ma, at a distance of approximately 79 cm to center of the rabbit, with .5 mm copper and 1 mm aluminum filtration, at a rate of approximately 43-45 r per minute. The rabbits received 800 r total body irradiation, ½ to each side† ($LD_{50/30}$).

The test microorganism was *Klebsiella pneumoniae*, Type A, grown 18 hours in brain heart infusion broth and diluted 1:1000 in saline. *The inocula* (1 ml of this dilution), as determined by plate counts, contained 5 x 10^5-10^6 microorganisms in most experiments, occasionally 2 x 10^6. Such inocula caused no deaths in normal rabbits. Groups of 8-20 rabbits, which usually included some unirradiated controls, were inoculated into the marginal ear vein. At intervals thereafter samples of blood for culture were obtained from one of the ear vessels not used for inoculation. Blood was drawn into a 2 ml syringe which had been rinsed with sterile heparin and 1 ml distributed as quickly as possible onto the surfaces of 5 nutrient agar plates and spread by means of sterile beads. When high bacterial counts were anticipated, additional plates were inoculated with saline dilutions of blood. Colony counts were made after 24 hours incubation at 37°C. In some experiments blood was cultured at 1, 2, 3, 4 and 12 hours after inoculation, in other experiments at 4, 8, 12, 24 and 48 hours, and in survivors at 120 hours. In one experiment (4 rabbits) bleedings were begun immediately after inoculation. The overlap in the 4-12 hour cultures accounts for the spread in the number of animals listed in the tables and figures. Final bleedings were sometimes made by cardiac puncture. Deaths among the irradiated rabbits reduced the number of bleedings which could be made. Leucocyte counts made on a number of rabbits before inoculation are presented in Table I.

Results. The rate of clearance of the inoculated bacteria during the first hour after intravenous injection is shown in Table II. Immediately (approx. 3 minutes) after inoculation of 10^6 *Kl. pneumoniae* the bacterial counts showed the circulating blood to con-

TABLE I. White Blood Counts before Inoculation.

	Leucocytes/mm³		No. rabbits
	Avg	Range	
Unirradiated controls	8320	4000-14500	15
2 days post-irrad.*	621	375- 710	5
3 " " "	310	100- 900	20
4 " " "	323	90- 730	17

* 800 r x-irradiation.

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* From a snuffle-free colony maintained by Mr. Harold H. Swift.

† The authors wish to acknowledge the services of Mr. James Bland and the facilities made available to them by the Argonne Cancer Research Hospital.

TABLE II. Rate of Disappearance during First Hour.

Min. after inoculation*	No. Klebsiella/ml blood Controls		 Irradiated†	
3	4000-7500		3010-4275	
10			32-	26
15-20	4-	15	1-	3
30	0	0	0	0
60	0	0	0	0

* 10^6 *Kl. pneumoniae* i.v.
† 3 days after irradiation with 800 r.

tain over 3,000 per ml of blood.‡ In both the irradiated (3 days after 800 r) and unirradiated control rabbits the counts fell to zero within the first half hour.

Table III shows that the counts were still zero, or nearly so, at the 3rd and 4th hours after inoculation in a much larger series of rabbits, including unirradiated controls and animals irradiated 1-8 days before inoculation. Some of these rabbits were bled at hourly intervals after inoculation and some for the first time at the 4th hour. Median counts per ml of blood are given in Table II, but they could not be used to describe the results of the later cultures because of the wider range of the counts. Instead, it was necessary to calculate the proportion of animals falling into 3 arbitrary categories; those showing <10, 10-50, and >250 bacteria per ml of blood. Fig. 1 presents the results on the cultures from the 4th hour on. It was in the rabbits inoculated 3 or more days after irradiation that the later (>8 hr.) counts showed the greatest difference from the unirradiated controls. In these animals the counts rose to more than 1,000 and in moribund animals to many thousands. All of the irradiated rabbits with rising counts, indicative of increasing bacteremia, died. Deaths occurred somewhat earlier among the rabbits in the 3-4 day group.

TABLE III. Clearance at 3rd and 4th Hours after Inoculation.*

	No. rabbits 3rd hr	 4th hr	Bacteria/ml blood† 3rd hr	 4th hr
Unirrad. controls	11	38	0	0
1 day post-irrad.	4	23	0	0
2 days ” ”	14	34	0	0
3-4 ” ” ”	6	44	0	3
6 & 8 ” ” ”	28	46	1	1

* 5× 10^5-10^6 *Kl. pneumoniae* i.v.
† Median observation.

No deaths occurred among the unirradiated rabbits. Because of lack of space, it was necessary to discard all but 28 of these control animals before the final bleeding at 120 hours. Plate counts at this time were all negative.

Discussion. The results demonstrate that during the first few hours after a single intravenous injection, irradiated (800 r) rabbits were able to remove *Kl. pneumoniae* from the circulating blood as effectively as unirradiated controls. In some of the latter, bacteria in moderate numbers reappeared in the blood but disappeared again before the 120th hour, a phenomenon observed by Wright(8) and others.

By contrast, in most of the rabbits inoculated 3 or more days after irradiation, the initial reduction in numbers of bacteria in the blood was followed by a rapid increase which terminated in death. It is evident, therefore, that the irradiated rabbits were able to clear their blood as effectively as the unirradiated animals but were unable to prevent the subsequent bacteremia. In other words, irradiation had not affected the filtration mechanism but had seriously impaired its capacity to retain and destroy the bacteria which had been removed from the circulating blood. Although no histological studies were made on these rabbits, it is clear from the work of Cannon(9) and others that removal of the bacteria is accomplished chiefly by the reticulo-endothelial system. To be sure, Wood (10) and his co-workers have recently called attention to the role of the phagocytic activity of the circulating leucocytes within the capillaries of the reticulo-endothelial system. However, in our irradiated rabbits this system received practically no assistance from circulating leucocytes because of the post-irradiation leucopenia. Moreover, Kerby and Martin(11) have shown that in rabbits made leucopenic by benzene or metchlorethamine HCl,

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‡ These counts at 3 minutes are consistent with estimates based on blood volume taken as 5 per cent of body weight, the method used by Wright(8) who considered it impracticable to determine the initial concentration of bacteria because of the rapidity with which they were being removed.

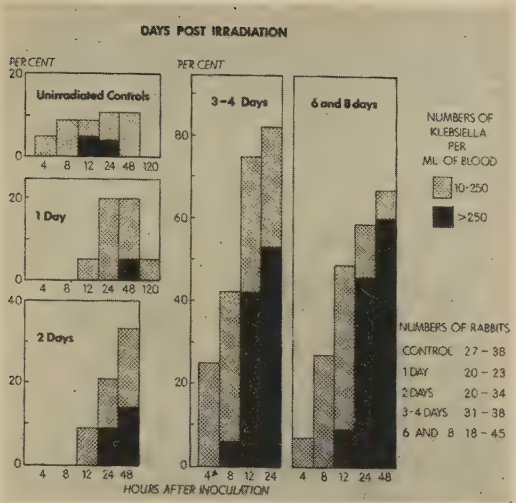

FIG. 1. Results of later blood cultures as percentage distribution of plate counts <10 (not shown), 10-250, >250.

* Height of this column gives an erroneous impression as the highest count in this group was 20/ml.

the splanchnic circulation was able to remove *Micrococcus aureus* introduced by continuous infusion.

Summary. Normal and irradiated rabbits were inoculated intravenously with approximately 10^6 *Klebsiella pneumoniae*, Type A. At intervals thereafter plate counts were made on peripheral blood. During the first 4 hours after inoculation the rate of clearance of microorganisms from the blood stream was as rapid and almost as complete in rabbits which had been exposed 1-8 days before with 800 r total body x-irradiation ($LD_{50/30}$) as in unirradiated controls. From the 8th hour on, in most of the rabbits 3-8 days post-irradiation, the colony counts increased steadily until the animals died. These findings indicate that in the irradiated rabbit the reticulo-endothelial system was able for a few hours to remove the bacteria from the circulating blood but was unable to retain and destroy them.

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2-Deoxyglucose—A Metabolic Block for Glucose.* (21881)

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It is generally agreed that insulin increases the intracellular transfer of glucose although the mechanism effecting the transfer is obscure. Ordinarily the glucose transfer rate is accelerated 2 to 4-fold by maximal insulin effect. This acceleration could be brought about by a change in the general permeability of the cell membrane to hexoses. However, insulin exerts its action on very few of the compounds which investigators have studied,

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TABLE I. Apparent Volume of Distribution of 2-Deoxyglucose in Control, and in Insulin Treated, Eviscerated Animals. Values expressed as % of body weight.

	Insulin treated				No insulin			
Exp. No.	R438	R432	R434	R430	R425	R433	R435	R429
2-deoxyglucose inj. (mg/kilo)	250		500		250		500	
Time, hr								
.5	23	28	41	40	36	30	31	28
1.0	34	36	52	46	43	32	33	30
1.5	42	39	54	56		52		
2.0	43	56	56	73	51	55	36	37
2.5		78		75		62		40
3.0	50	84	61	75	73	62	43	43
3.5		84						
4.0	66	90	63			62	50	
5.0	73	87	63			62		

indicating that the mechanism is rather specific in its action. Insulin accelerates the intracellular transfer rate of glucose, galactose (1,2), mannose(3), and glucosamine(4) in the eviscerated animal. It has no measurable effect on the closely related hexoses—sorbitol, fructose, 3-methyl glucose, gluconic acid, or glucuronic acid in this preparation(5). The action of insulin on compounds related to glucose in promoting entry into the cell is thus very limited and selective.

In a continuation of our studies of insulin action on glucose analogs, we have investigated 2-deoxyglucose, and the results are reported here.

Methods. Analytical Procedures. The 2-deoxyglucose was prepared as described by Bergman(6) and modified by Cramer(7).

The method used for determining the plasma concentration of 2-deoxyglucose is based on the observation that 2-deoxyglucose gives negligible reducing values in the Miller-Van Slyke(8) blood sugar method when the heating time is 8 minutes. The Miller-Van Slyke method gives maximum reducing value for glucose after 8 minutes heating in a boiling water bath, and no further reducing effect is observed when the heating is continued for 60 minutes. With deoxyglucose the maximum reducing value is obtained only after a 60-minute period, corrected for the glucose concentration as determined by the 8-minute determination.

Treatment of animals. Non-fasted rabbits, eviscerated and nephrectomized as previously described(9), were maintained at a normal blood sugar concentration at all times. In those experiments in which radioactive glucose (uniformly labeled with C^{14}) was used, a primer dose of sugar of high radioactivity was administered at the start of the experiment in order to adjust the radioactivity of the circulating plasma glucose to that of the sugar to be used for the continuous injection. The expired CO_2 was collected and its radioactivity determined as described in other reports (10). When insulin[†] was given, it was injected intravenously in maximum amounts, *i.e.*, over 5 units per kilo repeated each 30 minutes.

Results. Table I shows the effect of insulin on the volume of distribution of injected 2-deoxyglucose, the results expressed as percent of the body weight. Without insulin the injected 2-deoxyglucose distributes quickly in a volume equivalent to the extracellular compartment and passes rapidly from there into the cells. Samples taken at 30 minutes after injection give volumes of distribution greater than that of the extracellular compartment, (except in Exp. 1) which indicates that some intracellular transfer had already taken place at this time. (We consider the extracellular compartment to be equivalent to 20-25% of the body weight.) It is apparent that 2-deoxyglucose can enter the extrahepatic cells at a rather rapid rate in the absence of insulin.

With insulin administration there appears

[†] The insulin used in this work was contributed by the Lilly Research Laboratories.

TABLE II. Effect of a Single Injection of 2-Deoxyglucose on Intracellular Transfer and Oxidation Rates of C^{14} Labeled Glucose in Insulin Treated Animals. Oxidation results expressed as % of expired CO_2 from injected glucose.

Exp. No.	R430	R432	R440	R439	R442	R441	R285*	R286*
Dose of 2-deoxyglucose (mg/kilo)	500	250	150	100	50	Control	Control	Control
1 hr	3	6	—	—	—	—	11	6
2	5	11	—	—	—	—	27	16
3	8	13	—	—	—	—	37	22
4	10	15	10	7	—	—	39	30
5	13	20	14	9	23	29	43	39
6	14	—	14	12	26	33	39	39
7	16	—	16	14	30	35	43	42
8	—	—	17	14	35	38	43	45
Glucose disapp. (mg/kilo/hr), avg 8-hr period	165	220	155	238	290	470	457	440

* Data taken from previous publication(12).

to be an increase in the rate of entry of 2-deoxyglucose into the cells; however, positive conclusions on the insulin effect must depend on data obtained using 2-deoxyglucose tagged with C^{14} so that smaller amounts of the sugar can be administered, or on data obtained from more sensitive chemical methods for the determination of 2-deoxyglucose.

Table II shows the effect in insulin treated animals of a single injection of 2-deoxyglucose on subsequent oxidation of glucose and on glucose transfer rate. 2-deoxyglucose administration markedly diminishes the amount of glucose needed to maintain a normal blood sugar level (100 mg%). Inhibition of glucose transfer was not a temporary effect but was uniform throughout the experimental period. Insulin treated animals require on the average 450 to 500 mg/kilo of glucose per hour(10), whereas the glucose requirement for those given 2-deoxyglucose in addition is definitely reduced in amounts proportional to the dose of the deoxy compound given. The reduction in glucose transfer is accompanied by a low rate of glucose oxidation.

A dose of 150 mg of 2-deoxyglucose reduced the intracellular transfer rate of glucose to that which is shown by the eviscerated rabbit not given insulin. This blocking is probably more complete than indicated by the figures since undoubtedly much of the glucose that leaves the plasma in these preparations passes into special cells where the entry is not mediated by insulin, *i.e.*, brain and red blood cells. The amount of glucose oxidized by the insulinized animals given deoxyglucose is about the same as that oxidized by eviscerated rabbits not given insulin. Most of the glucose oxidation in the deoxyglucose-insulin treated animals and in control eviscerated animals probably takes place in tissues that are not dependent on insulin for transfer of glucose. We have previously summarized evidence suggesting that the changes in oxidation rate of glucose are secondary to changes in the transfer rate(5). We did not observe a decrease in CO_2 output in the animals injected with 50 to 150 mg/kilo of 2-deoxyglucose. Large quantities such as 500 mg did unquestionably lower the animal's metabolic rate.

Discussion. 2-deoxyglucose rapidly enters the cells of the extrahepatic tissues in the absence of insulin and insulin administration appears to increase the rate of cell entry. The intracellular transfer of this glucose analog inhibits glucose transfer and consequent oxidation. 2-deoxyglucose may then be considered, as a result of its action, a metabolic block for glucose. In this respect it resembles glucosamine(4) although 100 mg/kilo of 2-deoxyglucose will block glucose transfer as effectively as 500 mg/kilo of glucosamine. Since the blocking effect is constant over an 8-hour period (longer times not examined), it would appear that 2-deoxyglucose is either not metabolized or is only slowly metabolized by the extrahepatic tissues. The use of the tagged form will facilitate further studies with this compound.

Insulin acts very selectively on certain compounds closely related to glucose. This suggests enzyme involvement. Since the first known step in the metabolism of glucose involves the hexokinase system, it is of interest that Sols and Crane(11), in a study of substrate specificity of brain hexokinase, have stated as follows: "The phosphate ester formed from 2-deoxyglucose is not inhibitory, and it is not a substrate for either phosphohexose-isomerase or glucose-6-phosphate dehydrogenase. Thus, the use of 2-deoxyglucose isolates the hexokinase reaction." Our results with this compound in the living animal could be explained as an inability of the extrahepatic tissues to metabolize the phosphate ester of 2-deoxyglucose.

Summary. 2-deoxyglucose rapidly enters the cells of the extrahepatic tissues in the absence of insulin. Insulin appears to accelerate the intracellular transfer. The intracellular transfer of 2-deoxyglucose inhibits the transfer and oxidation rates of glucose, thus acting as a metabolic block for glucose. It is suggested that the blocking of glucose may take place at the 6-phosphate stage.

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Protection Against Diabetogenic Action of Alloxan.* (21882)

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The mechanisms whereby certain chemical compounds block the diabetogenic action of alloxan are becoming clearer. At least 2 important modes of action must be taken into consideration: A) the antagonist may reduce alloxan to some compound which is ineffective as a diabetogenic agent(1), B) the antagonist or its metabolites may form additional compounds or condensation products with alloxan which are ineffective in the production of diabetes(2,3). Many workers(3-7) have shown clearly that compounds containing sulfhydryl groups decrease alloxan-induced diabetes. In this latter regard the methemoglobin formers, which indirectly cause an elevation of blood sulfhydryl groups, would be included(8).

Although the above 2 chemical mechanisms are probably adequate to explain the protective action of most known antagonists of alloxan, it is also possible that certain agents decrease the flow of blood to the pancreas, thereby effecting a decrease in the quantity of alloxan reaching the islet cells(9).

Nicotinic acid(10) and nicotinamide(11) protect against the diabetogenic action of alloxan. It is not yet known whether these agents act through a direct chemical reaction with the latter compound. The present report is concerned with the protection effected

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TABLE I. Protection against Diabetogenic Action of Alloxan.

Chemical		No. rats	% diabetic	p value*
	mg/kg			
Alloxan (controls)	115	50	92	
” and nicotinic acid	125	31	74	.5
” ” nicotinamide	125	39	33	$<.001$
” ” acetamide	125	18	78	.5–0.3
” ” benzamide in water	125	24	4	$<.001$
” ” benzamide in propylene glycol	125	15	7	$<.001$
” ” N-cyclohexyl benzamide in propylene glycol	20	14	13	$<.001$
” ” N-cyclohexyl benzamide in 8% acacia	115	14	64	.2–0.1
” ” N,N-dicyclohexyl benzamide in propylene glycol	20	34	38	$<.001$
” ” N,N-dicyclohexyl benzamide in 8% acacia	50	15	93	$>.9$
” ” propylene glycol	4 cc/kg	16	19	$<.001$
	mg/kg			
” ” ethyl alcohol	100	9	89	.9–0.8
” ” sodium lactate	100	9	78	.7–0.5
” ” N-propyl alcohol	100	20	25	$<.001$
” ” isopropyl alcohol	100	20	55	.01–0.001

* These values were obtained by the χ^2 method based upon comparison of alloxan treated controls with the experimental situation under the null hypothesis.

against the diabetogenic action of alloxan by several amide compounds and certain simple alcohols. An abstract of some portions of the present investigation has already appeared (12).

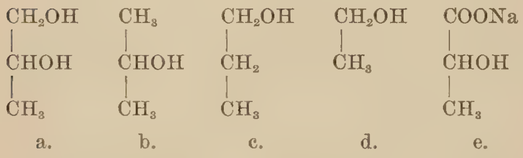
Methods. Adult male rats (328 in number) of the Long-Evans strain were fasted for 48 hours before they were given an intraperitoneal injection of a compound to be tested for antidiabetogenic activity. The alloxan, prepared as a 5% aqueous solution, was given subcutaneously. In some instances the test agents were given in doses (mg/kg) equivalent to the doses of alloxan used in the tests. It was necessary in the case of a few materials to reduce the dose because of their toxicity. The dose of alloxan was varied from 100 to 115 mg/kg body weight depending on the known diabetogenic activity of the particular batch of this material. The interval between the time the test substance was given and the alloxan was administered depended on the type of solvent or vehicle used in making it up. Alloxan was given 5 minutes following aqueous solutions, 30 minutes following compounds dissolved in propylene glycol and 90 minutes following the injection of certain benzamide derivatives that were suspended in gum acacia. All rats were given Rockland rat ration after the fast.

To be considered diabetic a rat had to show at least a 3+ qualitative urine sugar (Benedict's) 14 days following the injection of alloxan or a blood sugar value of at least 200 mg% by the method of Somogyi. In this screening procedure, data for animals which died within 72 hours following the injections were discarded. Rats which died thereafter were included as diabetics because they died of this disease and not from the acute toxic effects of the test agent.

Control rats, receiving alloxan alone, were used in each experiment.

Results. The subcutaneous injection of 115 mg of alloxan/kg body weight produced diabetes in 92% of the rats. However, it was possible to block this effect with certain amide compounds (Table I). Nicotinamide, benzamide when dissolved in water or propylene glycol, and N-cyclohexyl benzamide when dissolved in propylene glycol gave statistically significant protection. The propylene glycol was used as a vehicle because N-cyclohexylbenzamide was practically insoluble in water. When N-cyclohexyl benzamide was suspended in gum acacia and given in larger amounts it was ineffective in protecting against alloxan induced diabetes. Consequently, it was assumed the protection in this case was actually due to the solvent. Indeed, when propylene glycol *per se* was given before alloxan it afforded almost complete protection (Table I). Fifty mg/kg of N, N-dicyclohexyl benzamide in acacia failed to block the action of alloxan. As in the case of N-cyclohexyl benzamide, it did offer protection when dissolved in propylene glycol. Acetamide, an alkyl amide, in contrast to the aromatic amides of benzoic and nicotinic acids, did not offer protection.

Of particular interest was the fact that propylene glycol offered protection against the diabetogenic action of alloxan. In order to elucidate the mode of action of this compound, a number of related products were studied (Fig. 1). These included ethyl alcohol, sodium lactate, n-propyl alcohol, and isopropyl alcohol. Of these latter substances only the propyl alcohols offered protection (Table I).



$$\begin{array}{ccccc} CH_2OH & CH_3 & CH_2OH & CH_2OH & COONa \\ | & | & | & | & | \\ CHOH & CHOH & CH_2 & CH_3 & CHOH \\ | & | & | & & | \\ CH_3 & CH_3 & CH_3 & & CH_3 \\ a. & b. & c. & d. & e. \end{array}$$

FIG. 1. Structures of propylene glycol and related products. a. propylene glycol, b. isopropyl alcohol, c. n-propyl alcohol, d. ethyl alcohol, e. sodium lactate.

Summary. In the present study the following compounds offered protection against the diabetogenic action of alloxan: nicotinamide, benzamide, propylene glycol, N-propyl and isopropyl alcohols. The modes of action of these compounds are as yet unknown.

The nicotinic acid and nicotinamide used in this study were kindly supplied by Hoffmann-LaRoche, Inc.

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A Serologic Test for Identification and Titration of Vesicular Stomatitis Virus.[*] (21883)

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The standard complement fixation (CF) test utilizing homologous antigen and antibody (guinea pig hyperimmune serum), guinea pig complement, and sensitized sheep erythrocytes has proved to be a reliable *in vitro* method of identification and titration of vesicular stomatitis virus (VSV)(1). The CF test has, however, not been a satisfactory method of detecting antibodies in sera from bovines that have recovered from either vesicular stomatitis or foot-and-mouth disease.

In the early twentieth century it was ob-

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[*] Presented in part at the Conference of Research Workers in Animal Diseases of North America, Chicago, November 29-30, 1954. No abstracts of this conference are published.

TABLE I. Comparison of CF and GPEL Tests* with VSV Infected CAM Antigen.

Type of antigen	Type of guinea pig serum; 20% dilution	Type of test	Reciprocal of antigen dilutions						
			Undil.	2	4	8	16	32	64
NJ type virus	NJ Hyperimmune	CF	4	4	4	4	4	—	—
		GPEL	4	4	4	3	1	—	—
	Ind Hyperimmune	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—
	Normal	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—
Ind type virus	NJ Hyperimmune	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—
	Ind Hyperimmune	CF	4	4	4	4	4	2	—
		GPEL	4	4	4	4	—	—	—
	Normal	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—
Normal tissue	NJ Hyperimmune	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—
	Ind Hyperimmune	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—
	Normal	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—

* CF = Complement fixation test. GPEL = Guinea pig erythrocyte lysis test.

served that guinea pig erythrocytes could be agglutinated by heat-inactivated bovine serum. When fresh horse serum was added to this mixture lysis of the guinea pig cells would take place. This lytic action was prevented by the addition of homologous bacterial antigen and antibody, and the phenomenon was utilized to a limited extent in the diagnosis of glanders in horses. Agglutination of the red blood cells was observed in a positive test, while complete hemolysis occurred in a negative test(2). Based on these observations a test for the identification and titration of VSV antigens was developed.

Materials and methods. Serial 2-fold dilutions of stock 10% antigens of VSV infected chorioallantoic membrane and bovine tongue epithelium were made in 0.4 ml volumes in physiological salt solution. Normal tongue epithelium and chorioallantoic membranes were used as controls. Four-tenths ml of heat-inactivated guinea pig hyperimmune serum and 0.4 ml of fresh or frozen horse serum were added to each tube containing 0.4 ml of the antigen dilutions. The guinea pig hyperimmune serum had a complement fixing titer of 1:160 before dilution. Suitable controls of normal guinea pig, bovine, and horse sera and guinea pig cells were included in the tests.

The tubes containing the antigen, antibody, and horse serum were shaken and incubated in a water bath at 37°C for 30 minutes. After the incubation period 0.25 ml of normal bovine serum inactivated at 56°C for 30 minutes and one ml of a 1% suspension of washed guinea pig erythrocytes were added to each tube. The tubes were again shaken and incubated in a water bath for 5 to 30 minutes, depending on the complete hemolysis of the controls.

Results. A comparison was made between a standard complement fixation test and this so-called guinea pig erythrocyte lysis (GPEL) test for the identification and titration of VSV antigens. The test was observed for the presence or absence of hemolysis. A positive test showed no hemolysis while a negative test showed hemolysis. It has been observed that normal tissue antigens or heterologous antigen-antibody combinations do not inhibit hemolysis of guinea pig erythrocytes while homologous antigen-antibody combinations exhibit various degrees of inhibition of hemol-

TABLE II. Comparison of CF and GPEL Tests* with VSV Cow Tongue Antigen.

Type of antigen	Type of guinea pig serum; 20% dilution	Type of test	Reciprocal of antigen dilutions Undil.	2	4	8	16	32	64
NJ type virus	NJ Hyperimmune	CF	4	4	4	4	4	3	—
		GPEL	4	4	4	1	—	—	—
	Ind Hyperimmune	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—
	Normal	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—
Ind type virus	NJ Hyperimmune	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—
	Ind Hyperimmune	CF	4	4	4	4	—	—	—
		GPEL	4	3	1	—	—	—	—
	Normal	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—
Normal tissue	NJ Hyperimmune	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—
	Ind Hyperimmune	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—
	Normal	CF	—	—	—	—	—	—	—
		GPEL	—	—	—	—	—	—	—

* CF = Complement fixation test. GPEL = Guinea pig erythrocyte lysis test.

ysis depending upon the concentration of antigen and/or antibody. The results of representative tests are summarized in Tables I and II with degrees of hemolysis indicated in numbers decreasing from 4 (no hemolysis) to negative (complete hemolysis). The technic of the standard complement fixation test was essentially that described by Rice and McKercher(1).

Discussion. The data from this preliminary investigation yielded results which were specific in detecting New Jersey and Indiana types of VSV antigens. This technic gave slightly lower titers than the standard complement fixation test. Preliminary data on the application of this test to VSV antibody titrations of New Jersey and Indiana types of guinea pig hyperimmune sera have yielded equally specific and sensitive results.

A limited study on 2 titrations of foot-and-mouth disease virus using the GPEL test has shown this method to be as useful as with VSV.

It is not suggested that this method is a substitute for the standard complement fixation test, but it promises to be a useful test. It may be applicable to antigen-antibody systems which fail to either fix guinea pig complement or in which there is an anticomplementary action which can be avoided by the GPEL test. It has the advantages of being less time consuming and does not require the preparation, titration, or use of guinea pig complement and sheep red blood cell amboceptor. Refinements of the test are being pursued as well as applications to other infectious agents.

In the preparatory period before any virus agents were released for study in our laboratory interest had developed in the GPEL test. It was applied to the testing of bovine sera for brucella antibody. An antigenic system composed of brucella antigen and antibody, and a hemolytic indicator system composed of fresh normal horse serum, inactivated normal bovine serum and guinea pig erythrocytes was used in a study of brucella antibody in 75 bovine sera. The titers of 32 positive and 43 negative sera obtained by the use of the GPEL test compared closely with the titers obtained with the tube agglutination test.

Summary. A method is described for the identification and titration of vesicular stomatitis virus which involves the use of the virus, immune serum, fresh horse serum, heat-inactivated bovine serum, and guinea pig erythrocytes. The results obtained using this technic have been shown to be specific.

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Biophysical Characterization of the RI (RI-67) Viruses.* (21884)

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Viruses of the recently discovered RI (Prototype RI-67) family(1-4) have been shown to be a principal cause of the acute respiratory illnesses commonly classed as undifferentiated acute respiratory disease (ARD), primary atypical pneumonia (PAP) of the type unassociated with cold or *Streptococcus* MG agglutinins and exudative pharyngitis (1-5). These viruses propagate readily in HeLa (human epidermoid carcinoma) cells (6) in tissue culture and produce a cytopathogenic effect. Viruses of this family are heterogeneous antigenically(1,2,4,8-10) and may exist as latent infections in human adenoid or tonsil tissues(7,8). All of the agents of the group possess a common group-specific complement-fixing antigen(1,2,4,8-11). The present paper is concerned with certain of the biophysical properties of the RI virus particles and of the complement-fixing substance.

Materials and methods. Viruses. Respiratory disease virus strain RI-67(1,2), recovered from a patient with primary atypical pneumonia, and strain AD6, recovered by Rowe *et al.*(7,8) from human adenoid cells in tissue culture, were propagated for 2 or 3 days in HeLa cell cultures† in bottles (32 oz. prescription size) according to the method described previously(1). Chicken serum maintenance solution† was used to support the viability of the cells during viral growth and each bottle yielded approximately 34 ml of infected culture material.

Ultrafiltration. In preparing materials for ultrafiltration studies, the contents of 10 HeLa culture bottles infected with the RI-67 virus were centrifuged at 2500 rpm for 10 minutes in the horizontal head of an International #2 centrifuge and the sedimented cells were resuspended in 45 ml of the supernatant fluid. The cells were then disrupted by treating for 5 minutes in a Waring Blendor and the suspension was clarified by centrifuging at 2500 rpm for 10 minutes. The superfluent was further clarified by 2 successive centrifugations at 8000 rpm for 15 minutes in the #40 rotor of the Spinco Model L ultracentrifuge.‡ The final supernate was passed through a Gradacol§ membrane with an average pore diameter (APD) of 1550 mu and this constituted the starting material for the ultrafiltration experiments dealing with size of infecting viral particles which were carried out the same day. Material employed in the ultrafiltration experiments designed to determine the size of the RI-67 complement-fixing (CF) antigen was prepared from infected crude HeLa cultures homogenized in a Waring Blendor and stored frozen at —20°C. Before use, this crude culture material was thawed and the supernatant fluid obtained following centrifugation at 8000 rpm for 15 minutes in the #40 Spinco rotor was passed successively through 3 layers of S and S #595 paper, a 1550 mu APD Gradacol membrane and a 520 mu membrane. This preparation was stored overnight at 4°C and examined the following day.

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* Certain portions of this paper were presented before the 55th Annual Meeting of the Soc. of Am. Bact. on 9 May 1955 in New York, N. Y.

† The HeLa cell cultures and the culture solutions employed in these investigations were obtained from Microbiological Associates, Inc., Bethesda, Md.

‡ Spinco Division, Beckman Instrument Co., Belmont, Calif.

§ Obtained from Dr. F. Himmelweit, Wright-Fleming Inst. of Microbiology, St. Mary's Hospital Medical School, London.

In general, filtrations were performed as described by Elford(12), using membranes of varying pore diameter at room temperature and at a pressure of 8-10 psi of nitrogen. Three to 5 ml of fluid were passed through each membrane and the total volume was collected for testing. The time required for filtration varied from 23 to 110 minutes, increasing with decrease in pore diameter. The virus infectivity titrations were performed the same day; the CF antigens, which are stable, were stored at 4°C until tested 2 or 3 days after filtration.

Sedimentation. The cells collected from 19 HeLa culture bottles infected with the RI-67 agent were washed 3 times in 75 ml amounts of Hank-Simms solution† and homogenized in 45 ml of this solution in a Waring Blendor. This material was clarified by spinning at 2500 rpm for 10 minutes and the supernate was centrifuged again for 15 minutes at 8000 rpm in the #40 Spinco rotor. The superfluent was stored frozen at –70°C in aliquots in sealed glass ampoules until used; this storage period did not exceed 2 months. The concentration of virus in this suspension was not great enough to permit optical detection of the sedimenting boundary in the analytical cell. For this reason, the procedure of pseudo-velocity measurement in the separation cell proposed by Epstein and Lauffer(13) and modified by Breese and Briefs(14) was used to estimate the virus sedimentation coefficient. By this method a determination is made of the length of time required in a given gravitational field to reduce the infectivity titer of the suspension in the inner compartment of the separation cell to 1/10 or less that of the initial whole suspension. This reduction in titer is taken to be the point at which the moving virus boundary crosses the barrier of the cell and the sedimentation coefficient is computed according to the equation of Svedberg and Pederson (15). The virus particle diameter is computed from the s^{w}_{20} value by means of the standard equation for sedimentation of spherical particles. In the present computations, the density of the RI-67 virus in suspension was assumed to be 1.104 which is a figure given for the PR8 strain of human influenza virus(16). In the experiments, the contents of the inner and the outer compartments of the separation cell were removed in sequence immediately after centrifugation and the infectivity titrations of these fractions and of the whole fluid were performed immediately.

Virus purification and electron micrography. Purified preparations of RI-67 virus were obtained both from the infected culture fluid and from infected cells. For the former, the contents of 3 HeLa culture bottles infected with RI-67 agent were centrifuged for 25 minutes at 2500 rpm in the horizontal machine to remove the cells and the supernate was clarified further by spinning at 8000 rpm for 20 minutes in the #30 Spinco rotor. The superfluent was then passed successively through two 1550 APD Gradacol membranes. This filtrate was centrifuged at 24,630 rpm for 30 minutes in the SW39 head of a Spinco model E centrifuge and the pellet was resuspended in 1/15 the original volume in synthetic mixture 199.† The material was then passed through an ultrafine sintered glass filter and the filtrate was spun again at 24,630 rpm in the SW39 rotor for 30 minutes. This sediment was taken up in 1/100 the starting volume in mixture 199 and constituted the final preparation. For purification from cell extract, the washed cells recovered from 12 HeLa bottles infected with RI-67 virus were resuspended in 15 ml of mixture 199 and disrupted by shaking for 2 minutes with glass spheres‖ in a Mickle tissue disintegrator.¶ The cell extract was clarified by spinning at 4500 rpm for 15 minutes in the angle head of the Sorvall SS-1 centrifuge and the supernate was spun for 15 minutes at 7500 rpm in the SW39 Spinco rotor. The superfluent was then filtered successively through a 1550 APD Gradacol membrane and an ultrafine sintered

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‖ Minnesota Mining and Mfg. Co., St. Paul, Minn.

¶ H. Mickle, Hampton, Middlesex, England

TABLE I. Separation of RI-67 Infectious Particles and CF Antigen by Centrifugation.

Centrifugation (rpm)	Time (min.)	Fraction	Whole culture Infectivity	Whole culture CF	Cell extract Infectivity	Cell extract CF
None	None	Whole	10^{-2}	8*	10^{-3}	256
12,590	45	Supernate	10^{-2}	8		
		Sediment	10^{-2}	0		
24,630	45	Supernate	Neg.	8		
		Sediment	10^{-2}	0		
24,630	30	Supernate			Neg.	128
		Sediment			10^{-3}	2
39,460	45	Supernate	Neg.	8		
		Sediment	10^{-2}	Undiluted		

* Numbers are reciprocals of antigen titers; 0 = titer less than undiluted, the lowest dilution tested.

glass filter after which the filtrate was spun at 24,630 rpm for 30 minutes in the SW39 rotor. The sediment, which contained the virus, was suspended in mixture 199 to give 1/20 of the original volume of cell homogenate and examined by electron microscopy. One preparation of the latent AD-6 agent was obtained from infected cell extract by the same procedure except that filtration through medium and fine sintered glass filters was substituted for the 1550 mu APD Gradacol membrane. Additionally, materials were prepared from non-infected HeLa culture fluids and cells in essentially identical fashion for control purposes. Samples collected at each successive step in purification of virus preparations were examined electron microscopically and tested both for infectivity and for CF titer. The various fractions were prepared for electron microscopy by the agar pseudoreplica method(14,17). The specimens were shadowed with uranium at $\tan^{-1}0.33$ and electron micrographs were made from random areas of the screens using an RCA model EMU-2C instrument.

Viral infectivity titrations were performed in HeLa cell cultures in tubes by the same general procedure described earlier(1). Serial 10-fold dilutions of virus ranging from undiluted to $10^{-3.0}$ were inoculated in 0.1 ml amount into pairs of culture tubes. Where more precise quantitation was desired, intermediate $10^{-0.7}$, $10^{-1.7}$ and $10^{-2.7}$ dilution steps were added. The highest initial dilution of virus causing a definitive cytopathogenic effect (20% or more of the cells in the culture degenerated) within 3 days following inoculation was taken as the titer.

Complement-fixation titrations of the viral preparations were carried out as described earlier(1) using antiserum from a human case of RI infection.

Results. Separation of RI-67 virus from the CF antigen by centrifugation. Five ml

TABLE II. Infectivity and CF Titers of RI-67 Virus Suspension after Filtration through Gradacol Membranes.

Gradacol membrane filtrate, APD* in mu	Exp. 1 Infect. titer	Exp. 1 CF titer	Exp. 2 Infect. titer	Exp. 2 CF titer	Exp. 3 CF titer
1550—Starting material	10^{-2}	32†	10^{-2}	32	16
210	10^{-1}	16	10^{-2}	16	ND‡
190	10^{-1}	16	10^{-1}	8	ND
160	Undiluted	8	Undiluted	4	ND
140	Neg.	8	Neg.	4	8
110					8
82					2
79					2
55					Neg.
46					"

* APD = Avg pore diameter. † Numbers are reciprocals of antigen titers.
‡ ND = Not done.

TABLE III. Pseudo-Velocity Determinations with the RI-67 Virus.

Velocity, rpm	Effective sedimentation time, sec.	Calculated $s^{w}_{20} \times 10^{-13}$ sec.	Infectivity titer: Whole suspension	Infectivity titer: Inner chamber	Infectivity titer: Reduction	Calculated particle size, mu
12,590	660		10^{-2}	10^{-2}	None	
”	900		10^{-2}	$10^{-1.7}$	$10^{0.3}$	
”	1140	680	10^{-2}	10^{-1}	$10^{1.0}$	115
”	1380	539	$10^{-2.7}$	$10^{-1.7}$	$10^{1.0}$	103
8,360	1560		10^{-2}	10^{-2}	None	
”	1980		10^{-2}	$10^{-1.7}$	$10^{0.3}$	
”	2280		$10^{-2.7}$	10^{-2}	$10^{0.7}$	
”	2520		10^{-2}	$10^{-1.7}$	$10^{0.3}$	
”	2760	667	10^{-2}	$10^{-0.7}$	$10^{1.3}$	114
”	3000	661	10^{-2}	10^{-1}	$10^{1.0}$	113

samples of the low speed supernatant fluid obtained from a triturated whole culture of RI-67 virus in HeLa cells or from the extract of a disintegrated infected cell suspension were spun for 30 or 45 minute periods in the SW39 rotor of the Spinco centrifuge at the speeds indicated in Table I. Following centrifugation, the upper 3.5 ml of superfluent of each sample was removed for testing and the sedimented substance, following removal of the remaining supernatant fluid, was resuspended in the original volume (5 ml) of chicken serum maintenance solution. The supernate and resuspended sediment were tested for CF and viral activity with the results shown in Table I. It is seen that the viral infectivity was reduced by centrifugation at 12,590 rpm and completely removed from the supernate at 24,630 rpm. By contrast, the CF activity of the fluid was not significantly diminished even after spinning at 39,460 rpm. These experiments showed that the antigen active in the CF test sedimented considerably less rapidly than the virus and was readily separated from it. The presence of small amounts of CF antigen in the 39,460 and 24,630 (infected cell extract) sediments suggests that some of the CF antigen may be particulate or absorbed on particles.

Characterization of RI viruses. Filtration. The experiments in which preparations of RI-67 virus were filtered through membranes of progressively smaller APD are summarized in Table II. The viral infectivity passed through membranes of 160 mu but not 140 mu APD. Based on the relationship between limiting porosity and particle size established by Elford(12), the probable diameter of the virus is 80-120 mu. The CF antigen, on the other hand, filtered through 79 mu APD membranes but not through 55; this indicates that a portion, at least, of the CF antigen does not exceed the 26-40 mu size range.

Sedimentation. Table III presents the s^{w}_{20} values for RI-67 virus obtained from a series of sedimentation experiments carried out at 12,590 and 8,360 rpm for time intervals which permitted determination of the minimal time required to effect a reduction of 90% or greater (at least 1 log) in virus titer of the inner compartment of the separation cell. The same stock virus suspension, stored in aliquots was employed throughout and the individual experiments were run on different days. In these studies, a 1 log reduction in titer was achieved in 1140 and 1380 sec. at 12,590 rpm and in 2760 and 3000 sec. at 8,360 rpm. The computed sedimentation coefficients for these time periods ranged from 539S to 680S and the diameter of the virus, calculated** for spherical particles in suspension was 109 mu (± 6 mu) for the s^{w}_{20} values.

Electron microscopy. Table IV shows that most of the virus present in the crude RI-67 culture fluid (Exp. 5) was recovered in the final purified fraction. However, approximately 95% of the infectivity was lost from

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** The equation used to calculate the virus diameter was: $r^2 = \dfrac{9\eta^{s}20}{2(\sigma - P)}$, where r = radius of the particles in centimeters, η = viscosity of the medium, σ = specific gravity of sedimenting particles, P = density of the medium. For the calculations, the virus density was assumed to be 1.104 and the particles were assumed to be of spherical shape.

TABLE IV. Infectivity and CF Titers of Suspensions of RI-67 Virus Purified from Infected HeLa Cultures.

			Material tested		Titer	
Virus	Exp. No.	Virus purified from	Fraction	Concentration	Infectivity	CF
RI -67	5	Infected culture fluid	Original	1×*	10^{-1}	ND
			Final	100×	10^{-3}	ND
RI -67	7	Infected cell extract	Original	1×	10^{-3}	256
			Final	20×	10^{-3}	2
AD- 6	14	" " "	Original	1×	10^{-3}	ND
			Final	10×	10^{-3}	ND

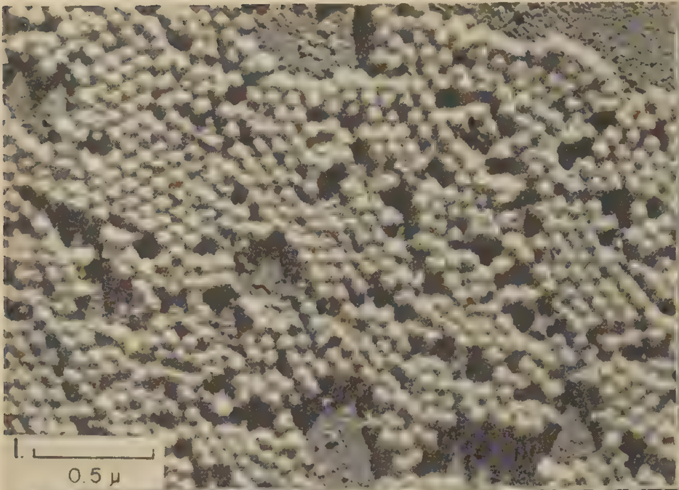
* Times concentrated in relation to original volume of material.

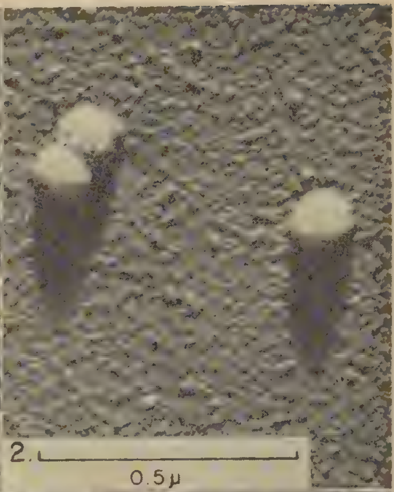
virus purified from the cell extract (Exp. 7). Titrations of infectivity performed at each step in the purification process revealed that essentially all of the loss of virus occurred during filtration through the ultrafine sintered glass filters. Similar loss occurred when the AD-6 virus was purified from the infected cell extract (Exp. 14).

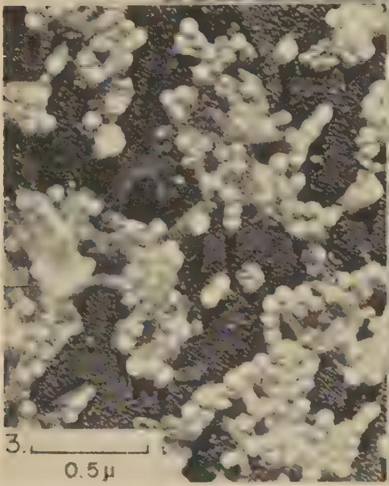
Electron micrographs of the RI-67 virus purified in Exp. 5 (Table IV) from infected culture fluid, reproduced in Fig. 1 and 2, revealed an abundance of spherical particles of remarkably uniform size and shape. The particles shown in Fig. 1 formed clusters in a regular array. Virus concentrated from extract of infected cells in Exp. 7 (Fig. 3) was of essentially identical size and shape to that recovered from the infected culture fluid but the preparation also contained a small amount of amorphous material. Measurement of 371 monodispersed particles from culture fluid and infected cell extract revealed a mean particle size of 90 mu with standard deviation of ± 4 mu; the diameter of the particles clustered in the arrays averaged 80 mu. The morphology of the spherical AD-6 virus particles purified in Exp. 14 and shown in Fig. 4 and 5 was similar to that of the RI-67 agent. However, flattened particles with depressed centers of about the same diameter as the virus were also present. A few such particles were seen in other preparations of the AD-6 and RI-67 viruses purified from the extract of infected cells; they were not observed in control materials prepared from uninfected HeLa culture.

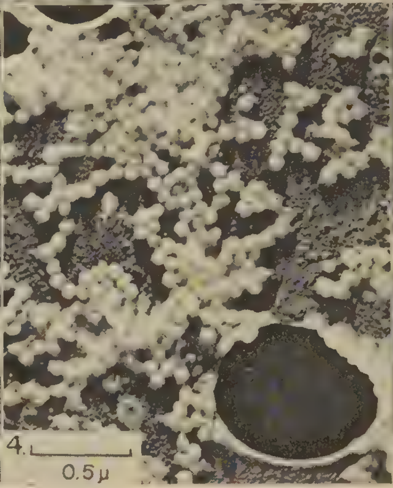
Discussion. Pseudo-velocity(13,14) determination of the sedimentation coefficient of viruses has the advantage that relatively small amounts of virus are necessary for measuring the moving boundary and that the boundary is definitively identified with the infectivity of the preparation. The sedimentation coefficients ranging from 539S to 680S obtained for the RI-67 virus at different speeds were in good agreement considering the limitations of the titration method resulting from the use of materials of low infectivity titer and the variability in susceptibility of tissue culture cells from day to day. The virus diameter values of about 109 mu (± 6 mu) computed from the sedimentation coefficient, of 80-120 mu based on the filtration data and of 90 mu (standard deviation ± 4 mu) determined by electron micrograph measurement were in close accord. The concurrence of the results of virus size determinations together with the observation that the profusion of 90 mu particles was found only in association with viral infectivity and not in non-infected HeLa culture material helped to identify the virus with these particles.

The indistinguishable morphology of the latent adenoid degenerative agent AD-6 from the RI-67 virus together with the sharing of a common CF antigen and other biological properties(1,2,7,8) provides a rationale for including the two in the same general family. No definitive conclusions can be drawn relative to the flattened particles with depressed centers seen in the electron micrographs of the purified RI viruses. It should be noted, however, that these particles were not found in non-infected HeLa cultures and bore a striking resemblance to the "doughnut" shaped precursor or immature particles of bacterio-









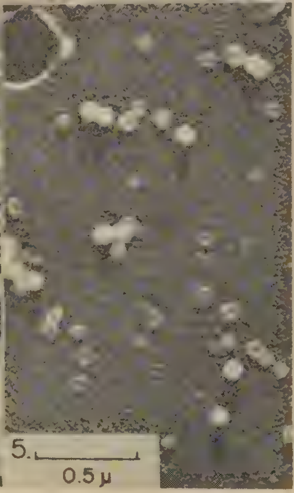


FIG. 1. RI-67 virus purified from infected HeLa culture fluid. 29,000×.
FIG. 2. Same as Fig. 1 showing individual particles. 72,000×.
FIG. 3. RI-67 virus purified from extract of infected HeLa cells. 29,000×.
FIG. 4. AD-6 virus purified from extract of infected HeLa cells. 24,500×.
FIG. 5. Same as Fig. 4 showing both spherical and flattened, "doughnut" shape particles.

phage which are deficient in nucleic acid and tails (See 18).

Kjellen *et al.*(19) and Harford *et al.*(20) describe the occurrence of "crystal-like" arrays of particles in nuclei of HeLa cells infected with RI viruses but not in nuclei of normal HeLa cells. The particles shown by Kjellen were approximately 50 by 30 mu in size and were separated from each other by a clear area measuring about 27 mu. If one assumes that the particles observed were but the central portions of larger particles which touched each other, the full particle size may be estimated to be around 77 by 57 mu. Particles of purified RI-67 in closely packed arrays, as shown in Fig. 1, measured about 80 mu, which is not greatly different from the 77 by 57 mu diameter for nuclear particles considering their probable tight aposition in the nucleus. Thus, while the identity of the nuclear particles is not known, their general size is not inconsistent with that of the viruses here discussed.

The numbers of individual spherical 90 mu particles observed in purified RI-67 virus preparations and the levels of CF activity (1:8-1:32) of homogenized whole cultures of the virus were much greater than anticipated for materials having infectivity titers of only 10^{-2} or 10^{-3}. Rough determinations[††] by electron microscopy of the numbers of virus particles in suspensions titering 10^{-2} or 10^{-3} were 5 x 10^7 and 1.5 x 10^9 per ml, respectively. The apparent discrepancy between infectivity titer and particle count suggests that an infectious unit, as estimated by the tissue culture method employed, contains many infectious particles or that much of the virus in the material is non-infectious.

The present study did not elucidate the nature of the CF antigen other than that it was of smaller size than the virus and that a portion, at least, did not exceed 26-40 mu in diameter. The observation that preparations free of detectable virus spun at high speed (24,630 or 39,460 rpm) contained a small amount of CF antigen suggested that the antigen may have been sedimented and was of particulate nature or was attached to particles in suspension.

Summary. The diameter of RI-67 virus in infected HeLa culture material was about 109 mu (± 6 mu) determined by pseudo-velocity sedimentation experiments, 80-120 mu based on Gradacol membrane filtration and 90 mu (standard deviation ± 4 mu) shown by electron microscope measurement. Purified preparations of RI-67 and latent adenoid degenerative agent AD-6 contained numerous spherical bodies of uniform size and shape and also a few "doughnut" forms. The CF antigen of infected RI-67 culture fluid sedimented less rapidly than the virus and passed through Gradacol membranes which held back the virus.

[††] By this procedure, 0.1 ml of the virus material was spread evenly over the surface of an agar block 0.4 sq. cm in area and each electron micrograph recorded the number of particles in an original volume of 6.25 x 10^{-8} ml of fluid. The particle counts were averages of 12 determinations for each virus sample.

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Inhibition of Experimental Neoplasms by 4-Aminopyrazolo(3,4-d)Pyrimidine.* (21885)

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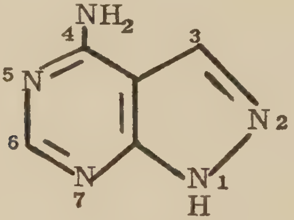
A considerable amount of effort has been directed toward production of purine antagonists(1) which have in turn been assayed for tumor-inhibiting and antileukemic activity in animals and man. At present the most useful of these agents from the standpoint of chemotherapy of acute leukemia in man is 6-mercaptopurine(1,2). Related compounds, thioguanine, 6-chloropurine, 6-methylpurine, and purine have been shown to inhibit growth of tumors in mice(3). It is also known that 2, 6-diaminopurine(4) and 8-azaguanine will inhibit growth of certain experimental neoplasms in mice(5). The present report is concerned with the observation of anti-cancer activity in experimental animals by a member of a new class of purine analogs, 4-aminopyrazolo(3,4-d)pyrimidine:



4-Aminopyrazolo(3,4-d)pyrimidine

Experimental. A description of the procedures employed for synthesis of the antagonists used in this study will be presented elsewhere(6). Since the mouse tumor Adenocarcinoma 755 is unusually sensitive to certain purine antagonists(7,8), initial screening of the pyrazolo(3,4-d)pyrimidines was carried out in mice bearing this tumor. The 755 tumor initially arose spontaneously as a mammary adenocarcinoma in C57 black mice, and has retained its strain specificity. The C57 black mice used in these studies were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. Males and females weighing 15-18 g were employed and were fed a laboratory chow diet. In all experiments treated groups of 10 animals each were used together with a group of 10 control animals receiving only the suspending agent, carboxymethylcellulose solution. Treatment was initiated at 24 hours following tumor transplantation, and continued every other day for a total of 6 injections. On the 12th day following tumor implantation (by the usual trocar method) all mice were sacrificed and the individual tumors were excised and weighed. Since growth of Adenocarcinoma 755 is inhibited by caloric restriction when this is sufficient to cause significant weight loss in the host(9), tumor inhibition in groups of animals in which treatment was accompanied by a loss of 10% or more of body weight was considered of questionable significance. The dosage levels employed were selected on the basis of preliminary screening trials and approach the maximum tolerated levels.

The effectiveness of 4-aminopyrazolo(3, 4-d)pyrimidine and 1-methyl-4-aminopyrazolo(3,4-d)pyrimidine in inhibiting the growth of Adenocarcinoma 755 is indicated by the data summarized in Table I. Also included in Table I are statistical data indicating the degree of significance of the difference between the control and treated tumor weights in each experiment.

Experiments were then carried out to determine the effectiveness of the two pyrazolopyrimidines in prolonging life span of mice with two strains of transplantable mouse leukemias. Leukemias L1210 and L5178 obtained from Dr. L. W. Law were used in dba mice purchased from Jackson Memorial Laboratory. In these experiments leukemic cells

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TABLE I. Effects of Two Pyrazolopyrimidines on Growth of Adenocarcinoma 755.

Compound	Dosage level (mg/kg)	Avg mouse wt change (g)	Mortality	Tumor wt (mg)		Avg tumor wt, % of controls	P
				Avg	Range		
4-Aminopyrazolo(3,4-d) pyrimidine	Control	+2.1	0/10	707	210- 986	—	—
	50 × 6	−2.3*	2/10	39	26- 52	5	<.001
	Control	+1.4	0/10	930	220-2510	—	—
	40 × 6	−2.4*	1/10	38	10- 82	4	<.001
	Control	+5.3	0/10	649	162-1306	—	—
	30 × 6	− .3	0/10	69	16- 140	11	<.01
	Control	+2.1	0/10	707	210- 986	—	—
	25 × 6	−1.0	0/10	41	12- 130	6	<.001
	Control	+1.4	0/10	930	220-2510	—	—
	20 × 6	− .7	1/10	150	58- 384	16	.01
	Control	+5.3	0/10	649	162-1306	—	—
	20 × 6	+ .1	0/10	103	40- 250	16	<.01
	Control	+2.1	0/10	707	210- 986	—	—
	15 × 6	−1.1	2/10	37	10- 76	5	<.001
	Control	+3.3	0/10	816	320-1070	—	—
	10 × 6	+2.6	0/10	123	20- 560	15	<.001
	Control	+1.4	0/10	930	220-2510	—	—
	10 × 6	− .5	1/10	262	52- 610	28	<.01
1-Methyl-4-aminopyrazolo(3,4-d)pyrimidine	Control	+3.3	0/10	816	320-1070	—	—
	100 × 6	−1.4	6/10	18	16- 20	2	<.001
	Control	+1.4	0/10	930	220-2510	—	—
	60 × 6	−1.8	5/10	38	14- 64	4	<.01
	Control	+2.1	0/10	707	210- 986	—	—
	50 × 6	− .4	0/10	61	10- 216	9	<.01
	Control	+5.3	0/10	649	162-1306	—	—
	50 × 6	+2.8	0/10	186	88- 314	28	<.001
	Control	+2.1	0/10	707	210- 986	—	—
	40 × 6	− .3	0/10	58	6- 326	8	<.001
	Control	+5.3	0/10	649	162-1306	—	—
	40 × 6	+2.8	0/10	130	24- 316	20	<.01
	Control	+1.4	0/10	930	220-2510	—	—
	20 × 6	+ .9	0/10	445	108- 778	48	.1

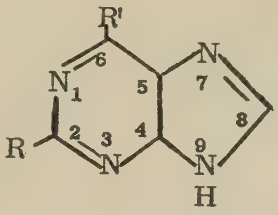
* Excessive weight loss.

Treatment was initiated (at the level indicated) at 24 hr after tumor implantation and continued every other day for a total of 6 injections. All tumors were weighed individually on the 12th day. Ten animals were used in each experiment.

were inoculated I. P., and therapy was initiated 24 hours later and continued every other day for a total of 10 injections or until death of the mice. Concurrent control and treated groups of 10 mice each were employed uniformly. The results of these trials, along with indications of their statistical significance, are summarized in Table II.

Discussion. The data presented in Table I indicate that 4-aminopyrazolo(3,4-d)pyrimidine and 1-methyl-4-aminopyrazolo(3,4-d)-pyrimidine are rather profound inhibitors of the growth of Adenocarcinoma 755 at levels which are not excessively toxic to the host. Only the 4-amino derivative significantly increased life span of mice with L1210 leukemia. In preliminary experiments both of the pyrazolo pyrimidines appeared to be active against leukemia L5178.

The purine derivatives which have been shown to possess temporary anticancer activity in mammals may be represented as follows:



R = H $\quad$ R' = H, SH, Cl, CH_3

R = NH_2 $\quad$ R' = NH_2, SH

It has also been shown that substitution of

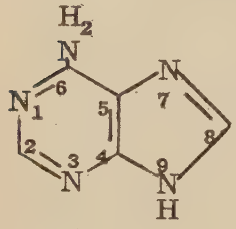
TABLE II. Effects of Two Pyrazolopyrimidines on L1210 and L5178 Leukemia in Mice.

Compound	Leuk.	Dosage (mg/kg)	Avg life span (days) Control	Treated	% above control	P
4-Aminopyrazolo(3,4-d)-pyrimidine	L1210	50	7.0	9.0	+28	<.001
		40	7.0	8.6	+23	.2
		30	7.0	8.1	+16	.02
		30	6.0	8.4	+40	<.01
		20	7.4	8.3	+12	<.02
		15	7.5	8.9	+18	<.2
		10	7.5	7.5	0	
	L5178	30	18.5	27.4	+48	<.001
		25	21.0	36.4	+73	<.001
1-Methyl-4-aminopyrazolo-(3,4-d)pyrimidine	L1210	50	7.5	7.9	+ 5	.2
		50	7.4	8.1	+ 9	.3
	L5178	40	18.5	>31.9	>+72	—
		40	21.0	>34.6	>+64	<.01
		25	21.0	22.6	+ 8	.3

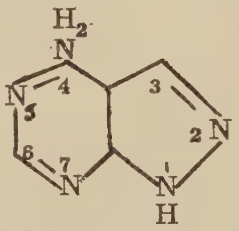
Treatment initiated 24 hr after inoculation of leukemic cells and continued every other day for a total of 10 injections or until death.

a nitrogen atom for the carbon atom at position 8 in the purine skeleton when R = NH_2 and R′ = OH results in an active tumor-inhibiting compound, 8-azaguanine.

In the present study it has been shown that rearrangement of the nitrogen atoms of the imidazole ring of the purine skeleton results in a compound with tumor-inhibiting activity and antileukemic activity. 4-Aminopyrazolo-(3,4-d)pyrimidine is an isomer af adenine:



Adenine



4-Aminopyrazolo(3,4-d) pyrimidine

It is somewhat surprising to observe that 1-methyl - 4 - aminopyrazolo(3-4-d)pyrimidine significantly inhibits tumor growth since one might assume that the 1-nitrogen of pyrazolopyrimidines would have to remain unblocked for ribosidation prior to competition with purine nucleotides.

A number of substituted pyrazolo pyrimidines have now been prepared and are being assayed for anticancer activity in mice. Clarke and Stock have observed that compounds in this series inhibit the growth of Sarcoma 180(10). Law has noted that certain of the pyrazolo(3,4-d)pyrimidines possess antileukemic activity in mice(11).

Summary. It has been demonstrated that 4-aminopyrazolo(3,4-d)pyrimidine (an isomer of adenine), and the 1-methyl derivative of this compound both significantly inhibit the growth of Adenocarcinoma 755 in mice. The former compound significantly increases the life span of mice with L1210 or L5178 leukemia. These compounds represent a new class of purine analogs with significant anticancer activity.

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Action of Norepinephrine on Carotid Sinus Arterial Walls and Blood Pressure.* (21886)

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Experiments showed(1-6) that epinephrine or norepinephrine applied locally to the arterial wall of the carotid sinus provokes a marked reflex fall of the systemic arterial pressure in normal and hypertensive dogs. These reactions are due to a stimulation of the carotid sinus baroreceptors. Epinephrine induces a contraction of the carotid sinus arterial walls, an increase of their pressure-response, and a decrease of their distensibility (7,8). It was concluded from these experiments that the state of contraction and tension, and thus the resistance to stretch of the arterial walls where the carotid sinus baroreceptors are located, are the primary factors affecting these receptors which regulate and buffer reflexly the systemic arterial pressure. Wezler and Böger(9) and Wezler and Sinn (10) stated, however, that epinephrine while contracting the arterial wall does increase its pulsatile expansion. Kezdi(11,12) claimed that local application of epinephrine on the barosensitive arterial walls, also increases their pulsatile expansion and thus stimulates the baroreceptors and provokes a reflex fall of systemic arterial pressure. Modifications and increases of pulsatile expansion of the barosensitive carotid sinus walls, would thus be responsible for the effects on blood pressure of the local carotid sinus application of epinephrine and norepinephrine.

To establish the role of modifications of pulsatile expansion of the carotid sinus arterial walls in the reflex control of blood pressure by local carotid sinus application of norepinephrine, experiments were carried out that alter tension of the carotid sinus baroreceptive walls, without affecting their pulsatile expansion.

Method. Dogs were anesthetized with morphine - chloralosane. The vagi-aortic nerves were cut to limit the reflex control of systemic arterial pressure to the carotid sinus baroreceptors. The efferent vessels, internal and external carotid arteries and occipital arteries of both carotid sinuses were ligated. Both common carotid arteries were also ligated in the neck and the cephalic ends of the common carotids were opened. In these conditions, both carotid sinuses were empty and, thus deprived of pressure and pulsatile expansion, while their innervation was maintained. The systemic arterial pressure was registered from a femoral artery by a mercury manometer

Results. The systemic arterial pressure remained at a high level as a consequence of the suppression of arterial pressure in the carotid sinuses (Fig. 1; A.P.: 250 mm Hg). Two hundred micrograms of norepinephrine tartrate in 0.2 ml physiological saline was then injected in the periadventitial tissue of the carotid sinuses (1, Fig. 1). The systemic blood pressure fell immediately from the high level of 250 to 70 mm Hg. It remained at this low level. Section (2, Fig. 1) of both

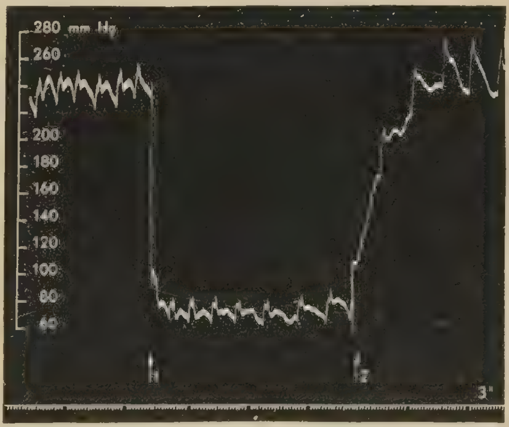

FIG. 1. Dog prepared according to described method. Systemic arterial pressure from a femoral artery with mercury manometer. 1: Local application to both carotid sinus arterial walls of 200 μg of norepinephrine. Marked fall of arterial pressure. 2: Section of both carotid sinus nerves. Marked rise of arterial pressure.

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* Supported by a grant from Belgian National Research Foundation.

carotid sinus nerves provokes an immediate rise of blood pressure from 70 to 260 mm Hg.

Discussion. These experiments provide evidence that local application of norepinephrine to the carotid sinus, which increases the state of contraction and tension of the arterial walls(7,8), leads to a marked stimulation of the baroreceptors, although neither pressure nor pulsatile expansion were present, nor could be altered in the carotid sinus. Changes in pulsatile expansion of the barosensitive arterial walls thus do not play a primary role in the development of baroreceptors stimulation and reflex fall of systemic arterial pressure induced by local carotid sinus application of norepinephrine. These experiments also confirm our previous conclusions that changes of tone and tension of the barosensitive arterial walls play a primary and fundamental role in the mechanisms of reflex regulation of blood pressure.

Summary. Local application of norepinephrine to the arterial walls of the carotid sinus induced a marked reflex fall of systemic blood pressure, although the carotid sinus walls were deprived of pressure and pulsatile expansion. Changes not in pulsatile expansion but in tone and tension of the barosensitive arterial walls play a primary and fundamental role in the mechanisms of reflex homeostasis of blood pressure.

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In vitro Incorporation of Iron-59 into Hemoglobin S Visualized by Autoradiography.* (21887)

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This investigation deals with the relationship between the initiation of hemoglobin (Hb) S synthesis and the stage of maturity of cells of the erythrocytic series. Walsh and associates(1) and Sharpe *et al.*(2) have shown that red blood cells take up Fe-59 for the synthesis of heme before they reach maturity. Thorell(3) on the basis of absorption microspectroscopy concluded that the initial appearance of Hb is in the rubricyte (polychromatic erythroblast). De Carvalho (4) using a more refined microspectroscopic technic demonstrated the presence of heme granules in the nuclei of prorubricytes (basophilic erythroblasts). Austone(5) using Fe-59 *in vitro* demonstrated its presence autoradiographically in nucleated red cells from rat bone marrow at all stages of maturation. The largest concentration was found in the rubricyte and the metarubricyte (orthochromatic erythroblast). By autoradiographic demonstration of Fe-59 uptake and subse-

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quent paper electrophoresis isolation of labeled Hb, we have evidence of *in vitro* Hb synthesis by immature erythrocytes from human bone marrow. This synthesis occurs at an earlier stage than heretofore suspected.

Method. A solution of 11 μg of ferrous chloride in 1 ml of 0.85% sodium chloride was labeled with Fe-59 and measured 1.14 x 10^7 counts per min. This was mixed with heparinized bone marrow obtained by sternal puncture from a patient with sickle cell anemia. The radioactivity value of the mixture was 1.5 x 10^6 counts/min/ml. Aliquots were placed in test tubes in an atmosphere of 5% oxygen and 95% nitrogen and were shaken at 37°C for 0.5 or 3.25 hr. Other aliquots were allowed to stand at 0°C for 3.25 hr. At the end of this time, the incubation mixture was centrifuged at 3000 RPM for 10 min. The cells were washed with 5 ml of 0.85% sodium chloride 3 times to remove extracellular iron. Autoradiograms were prepared according to the method of Boyd(6) using Kodak NTB plates (10 μ emulsion). Small aliquots of the washed cells were suspended in the patient's serum and smears were made on the plates in a dark room using a No. 2 Wratten safe light. The smears were fixed in methyl alcohol for 2 min, dried and placed in light-tight boxes at 7°C for 99 days. The plates were developed in Kodak D19b for 2 min at 20°C, washed in water, and fixed in fresh 10% sodium thiosulfate for 15 min at 20°C. The slides were washed in ice water for 30 min and stained with Wright's stain. In the remaining aliquot the cells were laked with water. Oxygen was bubbled through the mixture to oxygenate any reduced Hb. Toluene was added, the mixture was shaken vigorously and then centrifuged in a Sorvall high speed centrifuge. The hemolysate was removed and the Hb concentration, after suitable dilution, was determined in a Klett-Summerson photoelectric colorimeter. The radioactivity of the Hb solution was determined as counts/min/mg Hb. The hemolysate was subjected to fractionation by paper electrophoresis using 0.06 M Veronal buffer, pH 8.6, at 250 volts. The paper strip was divided into 16 mm sections and the radioactivity of each section was determined. A thin window Geiger-Mueller tube and scaler were used for all radioactivity measurements.

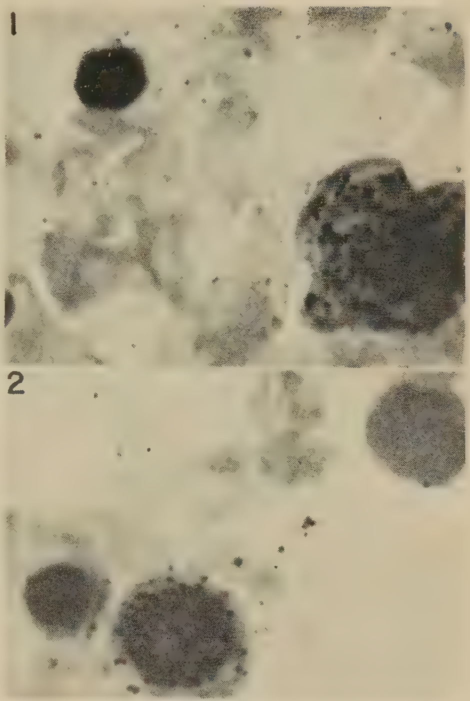

FIG. 1. Microphotograph (× 1000) of autoradiogram produced by sickle cell anemia bone marrow incubated with iron-59 for 0.5 hr at 37°C. 1. Rubriblast and metarubricyte. 2. Lymphocyte, prorubricyte and nonradioactive metarubricyte.

Results: The autoradiograms of bone marrow from a patient with sickle cell anemia show that after incubation with radioactive Fe for 0.5 hr at 37°C, radioactivity was present in all rubriblasts (proerythroblasts) and prorubricytes, in about 56% of the rubricytes and in approximately 24% of the metarubricytes (Table I and Fig. 1). Very few dif-

TABLE I. Percentage Distribution of Labeled and Non-labeled Cells of Erythrocytic Series after Incubation with Iron-59 at 37°C (400 Cells Counted on Autoradiograms). + means iron-59 located within the cell. — means no iron-59 located within the cell.

	Cell size	Incubated with iron-59			
		0.5 hr		3.25 hr	
		+	—	+	—
Rubriblast	12-15	7½	0	8½	0
Prorubricyte	10	13½	0	15	0
Rubricyte	8-9	18¾	13	42¾	0
Metarubricyte	6-7	11	36¼	33¾	0

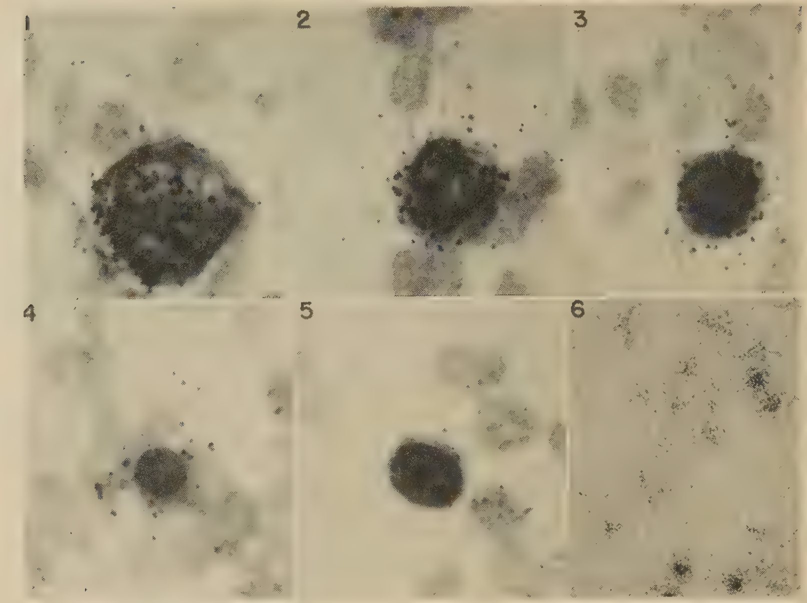

FIG. 2. Microphotograph of autoradiograms produced by sickle cell anemia bone marrow incubated with iron-59 for 3.25 hr at 37°C. 1. Rubriblast 2. Prorubricyte 3. Rubricyte 4. Metarubricyte 5. Polychromatophilic erythrocytes and lymphocyte (× 750) 6. Distribution of radioactive cells (× 200).

fusely basophilic (polychromatophilic) erythrocytes contained Fe-59.

All nucleated red blood cells contained radioactive Fe after incubation for 3.25 hr at 37°C. As visualized in Fig. 2, the major portion of Fe-59 in the rubriblast is found in or adjacent to the nucleus. In the prorubricyte the larger portion of Fe-59 is still present in the nucleus but this cell shows more Fe-59 in the cytoplasm than the rubriblast. The rubricyte and metarubricyte show a fairly equal distribution of radioactive Fe throughout the cell. A majority of the polychromatophilic non-nucleated erythrocytes were radioactive.

The aliquot of sickle cell anemia bone marrow incubated at 0°C for 3.25 hr produced no autoradiogram after 99 days exposure. There was no greater deposit of silver in the emulsion under the cells than that found in the background. Since mature eosinophilic staining red blood cells as well as leucocytes did not cause any blackening of the emulsion, one may conclude that the autoradiograms produced by the bone marrow incubated at 37°C were due to radiation effect on the emulsion and not due to chemical fogging.

Peripheral blood from a normal individual (Hb A) with a reticulocyte count of 0.8% was incubated in the same manner as bone marrow at 0°C or 37°C for 3.25 hr. The same amount of radioactivity (50-57 counts per min per mg of Hb *viz.* about twice background) was present in the hemolysates of washed cells when the cells had been incubated at 0°C as when the cells had been incubated at 37°C (Fig. 3). Since there was no appreciable radioactive Fe uptake by peripheral blood cells, autoradiograms were not attempted.

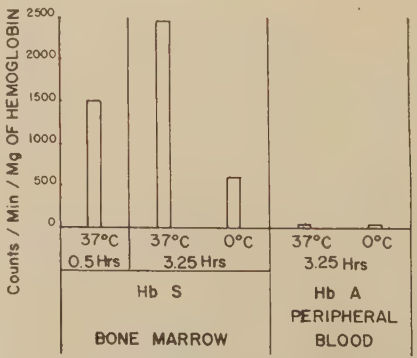

FIG. 3. Labeled hemoglobin synthesized during incubation of cells with iron-59.

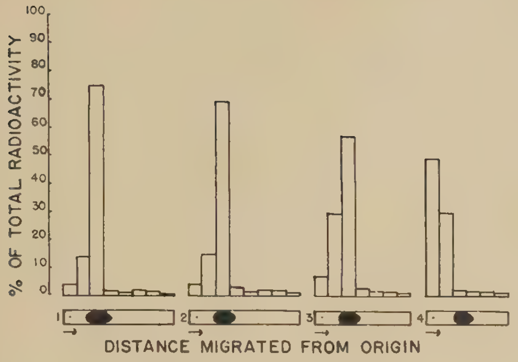

FIG. 4. Fractionation by paper electrophoresis of cell hemolysates after incubation of cells with iron-59. (1, 2, 3) Bone marrow from a sickle cell anemia patient. 1. Incubated 3.25 hr at 37°C. 2. Incubated 0.5 hr at 37°C. 3. Incubated 3.25 hr at 0°C. Each block represents a 16 mm section. 4. Peripheral blood from a normal adult incubated 3.25 hr at 37°C. Each block represents a 20 mm section.

Fig. 3 shows that the uptake of radioactive Fe by bone marrow cells containing only Hb S was rapid at 37°C since at 30 min the amount taken up was 62% of the 3.25 hr value. Reducing the temperature of incubation from 37°C to 0°C decreased the uptake of Fe-59 75%.

Addition of 0.02 M sodium fluoride did not alter the uptake of Fe-59 by immature erythrocytes at 0° or 37°C. This confirms the observation of Sharpe(2).

By means of paper electrophoresis it was demonstrated that 90% of the Fe-59 taken up by nucleated red blood cells of bone marrow was found in the Hb S fraction of cell hemolysates (Fig. 4). Hb of incubated peripheral blood subjected to the same procedure showed that the radioactive Fe did not migrate with the Hb; about 80% of the Fe-59 remained adjacent to the origin (Fig. 4).

Discussion: The autoradiograms (Table I, Fig. 1 and 2) show that the most immature nucleated erythrocytes from sickle cell anemia bone marrow took up radioactive Fe at a more rapid rate than the more mature nucleated cells. In autoradiograms the increased density of the silver grains around the youngest nucleated cells showed that these cells contained a greater amount of Fe-59 than did the older forms. The hemolysates of all these cells when fractionated by paper electrophoresis (Fig. 4) showed 90% of the total radioactivity present to be in the Hb fraction. It may be concluded that the earliest immature erythrocytes actively synthesize Hb in sickle cell anemia patients. The incorporation of Fe-59 into Hb by immature cells and not mature cells is clear evidence that the radioactive iron is used in the synthesis of hemoglobin and does not exchange with stable iron of Hb (Fig. 4).

Since autoradiograms showed that Fe-59 is concentrated in and around the nucleus of rubriblasts and prorubricytes and 90% of the radioactive iron is incorporated into the Hb, it is evident that Hb synthesis occurs in or adjacent to the nucleus. The technic employed in this investigation makes it possible to demonstrate Hb synthesis, in contrast to the observations of Knoll(7), Thorell(3), Metcalf(8) and de Carvalho(4) who demonstrated the presence of heme in the cell nucleus by means of cytochemical staining technics and absorption microspectroscopy. In the present study the presence of significant amounts of labeled Hb in rubriblast nuclei shows Hb synthesis at an earlier stage of erythrocyte development than assumed by these authors.

Summary. 1. Human bone marrow of a patient with sickle cell anemia and peripheral blood of a normal individual were incubated with iron-59 under similar conditions. Autoradiograms were prepared from the cells. After incubation, hemolysates of the cells were fractionated by paper electrophoresis and the radioactivity of the fractions was measured. 2. 90% of the total radioactivity was located in hemoglobin from bone marrow hemolysates. At 37°C the rate of hemoglobin synthesis as measured by iron-59 utilization is more rapid in the most immature erythrocytic cells beginning with the rubriblast. The rate of synthesis at 0°C was 25% of that at 37°C. 3. Less than 5% of the radioactive iron in incubated normal peripheral blood was in the hemoglobin fraction.

We wish to express our thanks to Dr. A. P. Kraus and Miss Ann Bell for checking the cell counts on all autoradiograms and to Dr. L. W. Diggs for ad-

vice on the hematological aspects of this investigation.

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Some Observations on Influenza Hemagglutination Inhibitor of Allantoic Fluid. (21888)

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Hemagglutination inhibitors for viruses of the influenza group are generally considered to be mucoproteins. The hemagglutination inhibitor present in normal allantoic fluid was investigated by Svedmyr(1,2), who obtained a sedimentation constant of about 200 x 10^{-13}, for the active fraction which showed chemical activity consistent with mucoprotein structure. However, after study of the allantoic fluid inhibitor, Neumuller(3) described a purified active fraction with a sedimentation constant of the order of 0.5 x 10^{-13}, and presented chemical evidence that it was a polysaccharide.

The present report deals with further attempts to isolate the inhibitor from the allantoic fluid and the application of the ultracentrifuge partition cell to the determination of the sedimentation constant of the active principle in the resulting fraction.

Materials and methods. Normal allantoic fluid from 50 to 100 eleven-day-old white Leghorn eggs was pooled for each experimental lot. The Lee strain of influenza virus was used in all experiments. Purified virus, containing approximately 8,000 CCA units per mg of nitrogen, was prepared from infected allantoic fluid by methanol precipitation(5) and differential centrifugation. CCA titrations were performed by the method of Hirst and Pickels(6).

Inhibition titers were obtained as follows: The inhibitor preparation was diluted in phosphate buffer, pH 6.9, ionic strength 0.2, in 2-fold serial dilutions of 0.5 cc each. To each tube was added 0.5 cc of indicator virus containing 4 to 6 CCA units. The mixture was shaken and allowed to stand 30 minutes at room temperature, then 1.0 cc of a 1.5% suspension of washed chicken red cells was placed in each tube and mixed by inversion. After agglutination had proceeded for 60-75 minutes, readings of the tubes were made in the Klett-Summerson colorimeter. A tube containing buffer in place of the inhibitor solution was used to determine the reading for no inhibition, and the end point was taken as the dilution of the last tube showing inhibition.

Sedimentation analyses were done in the Spinco model E centrifuge with inclined wire, using the synthetic boundary cell(7) to obtain sedimentation constants of slow moving fractions and the partition cell(4) to obtain the constant for the active principle, based on the ratio of activity in the "top" of the cell after centrifugation to that in the original preparation.

Experimental. Following Neumuller's procedure, it was not possible to obtain an active fraction of low molecular weight as measured by sedimentation in the partition cell. Accordingly, improved purification methods were devised, as outlined below.

1. *Acetone precipitation.* Freshly harvested allantoic fluid was dialyzed overnight against ion-exchange water, and to it was

TABLE I. Acetone Precipitation of Inhibitor (6 Batches).

Vol of allantoic fluid (cc)	H.I. titer of allantoic fluid	Ppt wt (mg)	Specific activity of Ppt (μg)*
150	1:256	103	31
223	1:256	68	15
280	1:256	120	15
296	1:256	125	8
333	1:64	108	8
250	1:128	90	8

* Based on H.I. titer using zero inhibition as endpoint. Activity yields ranged from 80 to 90%.

added magnesium chloride to a concentration of 0.01 molar. The fluid was maintained at 0-5°C, while an equal volume of cold acetone was slowly added. The resulting precipitate was centrifuged out, resuspended in water, and dried from the frozen state. The results for a series of 6 such precipitations, each representing fluids from 50 eggs, are shown in Table I. A sample of the powder obtained by acetone precipitation was dissolved in 90% phenol, the phenol extracted with chloroform after the addition of an equal volume of water, and the material recovered by lyophilization. More than 90% of the material in the resulting hygroscopic powder had a sedimentation constant of less than 1.0 and there were no appreciable rapidly sedimenting fractions. However, partition cell experiments indicated that the active principle had a sedimentation constant greater than 10.0. It was apparent that after treatment with 90% phenol only very small amounts of active inhibitor were present, and that the inhibitor still had a substantial molecular weight. Since phenol treatment was unsuccessful, it was thought desirable to attempt to purify the allantoic fluid hemagglutination inhibitor by other methods.

2. *Solubility in buffers.* The acetone precipitate was tested for solubility of the active fraction in a series of acetate and phosphate buffers ranging in pH from 4 to 7, and in ionic strength from 0.02 to 0.1. It was found that maximum solubility occurred with phosphate buffer pH 7.0, ionic strength 0.1. When the acetone precipitate was suspended in such a buffer at a concentration of 2 mg/cc and centrifuged for 15 minutes at 5000 rpm, some loss of activity resulted. However, the procedure removed such a large amount—60-75%—of inactive material that it was adopted as a step in purification after acetone precipitation.

3. *Adsorption on virus.* Further purification was next attempted. Following suspension in phosphate buffer and centrifugation as described above, to the supernatant were added 800-1,000 CCA units of indicator virus per cc. The mixture, after standing at room temperature for 30 minutes, was centrifuged for 45 minutes at 25,000 rpm in the #40 Spinco rotor. The pellet was resuspended in an equal volume of ion-exchange water, placed in boiling water for 3 minutes, cooled rapidly to room temperature, and again centrifuged at 25,000 rpm for 45 minutes. Table II gives colorimeter readings for a typical experiment of this type. Contrary to what might be expected, the specific adsorption of the inhibitor on the virus did not give a pure product. Ultracentrifuge analyses in the synthetic boundary cell indicated that at least 90% of the material was of low molecular weight, and that only a trace was of high molecular weight. Partition cell experiments still indicated that the activity of the inhibitor was associated with a substance of high molecular weight.

4. *Ultrafiltration.* Further purification of the material recovered from adsorption on virus was achieved by ultrafiltration. A 10% parlodion membrane supported on an unglazed porcelain thimble was used for filtration. The material was washed with saline

TABLE II. Absorption and Elution of Inhibitor from Indicator Virus.

Sample	Dilution									
	1:2	4	8	16	32	64	128	256	512	1024
Acetone ppt. in buffer, 2 mg/cc	900	600	385	340	290	(285)	210			
Supernatant after virus adsorption	155									
Supernatant after heating virus-inhibitor complex	520	470	325	290	275	(270)	230	230		

No inhibition—210. ( )—endpoint.

for 24 hours and then with distilled water for 24 hours. A small amount of cysteine was included to preclude any inactivation by oxidation. In this manner, it was possible to remove 95% of the material without loss of activity. The final product was active at about 0.5 μg and the activity had a sedimentation rate of 15. It was still grossly impure, showing a single peak with s = 1.43, and traces of heavier material.

Discussion. Although drastic procedures such as solution in 90% phenol and heating to 100°C degraded most of the material present in active hemagglutination inhibitor fractions recovered from allantoic fluid, the inhibitor was not degraded. In the allantoic fluid, the active principle appeared to have a sedimentation constant of 32. Precipitation with acetone caused some degradation, and the sedimentation constant dropped to 15. Treatment with phenol or heating to 100°C did not change this value. The hemagglutination inhibitor is present in allantoic fluid in very small amounts. Since a highly impure fraction was active at 0.5 μg, it is probable that the allantoic inhibitor has at least as high a specific activity as the urinary inhibitor of Tamm *et al.*(8).

Summary. Treatment of the hemagglutination inhibitor from allantoic fluid with 90% phenol did not result in a low molecular weight active substance. After acetone precipitation, adsorption on indicator virus and ultrafiltration, the partially purified inhibitor was active at 0.5 μg and the active principle had a sedimentation constant of 15.

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Effect of Metabolic Inhibitors on Production of CO_2 and H_2S by *Endamoeba histolytica*.* (21889)

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We have previously reported(1-3) that washed suspensions of *E. histolytica,* treated with certain precautions(2,3), exhibit a rapid rate of CO_2 and H_2S evolution when incubated under anaerobic conditions with glucose and cysteine (or cystine). The mechanism of this reaction was further studied(3) and it was concluded that in *E. histolytica* triose phosphate is oxidized enzymatically by cystine. The resulting cysteine is further oxidized by a reactive form of sulfur, which is formed by the desulfuration of a thioketoacid (β-thiopyruvate). As the result of this oxidation S is reduced to H_2S and cystine regenerated. Since this pathway of anaerobic oxidation is believed to play an important role in the intermediary metabolism of *E. histolytica,* it was considered to be of interest to test the effect of inhibitors on these enzymatic reactions.

Materials and methods. The preparation of washed suspensions of *E. histolytica* and the optimal conditions for metabolic measure-

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TABLE I. Effect of Inhibitors on CO_2 and H_2S Evolution.

No.	Inhibitors	Substrates	Enzyme activities*	
			CO_2	H_2S
1.	"Antibiotics"	glucose + cysteine	67	30
	"	" + cystine	91	14
	"	" + $Na_2S_2O_3$(10^{-2}M)	78	67
	"	" + glutathione	79	100
	"	" + –θ–	86	60
	"	–θ– + cysteine	100	4
2.	NaCN (10^{-3}M)	glucose + cysteine	40	40
3.	KF (1.6×10^{-2}M)	*Idem*	38	36
4.	NaN_3 (10^{-3}M)	"	64	60
5.	Na_2HAsO_4 (2×10^{-3}M)	"	39	100
6.	α,α′-Bipyridyl (10^{-3}M)	"	35	43
7.	8-Hydroxyquinoline (10^{-3}M)	"	21	44
8.	Chel-242-L (0.01 ml)	"	57	49
9.	Ortho-aminobenzoate (3×10^{-4}M)	"	100	60
10.	Para-aminobenzoate (3×10^{-4}M)	"	100	95
11.	Isonitrosoacet-paraaminobenzoate (3×10^{-4} M)	"	83	100
12.	2,4-Dinitrophenol (10^{-3}M)	"	50	89
13.	Pyocyanine (3×10^{-4}M)	"	65	90
14.	6-Bromo-2-nitrobenzoate (3×10^{-4}M)	"	87	115
15.	2,3-Diaminophenazine (10^{-5}M)	"	36	132
	"	pyruvate + cysteine	90	75
	"	–θ– + cysteine	0	50
16.	2-Amino-3-hydroxyphenazine (10^{-5}M)	glucose + cysteine	47	94
17.	Janus-green B (10^{-5}M)	*Idem*	50	93
18.	Malachite green (10^{-5}M)	"	66	85
19.	Methylene blue (3×10^{-4}M)	"	66	100
20.	Fumarate (2×10^{-2}M)	"	100	88
21.	Maleate (2×10^{-2}M)	"	86	54
22.	O_2 (100%)	"	–θ–	21

* Per cent activities, as compared to the controls, containing no inhibitors, but the same number of amoebae and substrates.

ments *in vitro* have been described(2,3). In the current study protozoa of clone 22(4), grown in aqueous egg yolk infusion medium (5), were used after centrifugal separation and one washing with the suspending medium (1,2,3). The analytical method for H_2S as well as the composition of the suspending salt solution was described previously(2,3,6); CO_2 originating from the enzymatic decarboxylation of pyruvate (formed from glucose) was determined manometrically. All reactions were carried out in an atmosphere of 95% N_2 + 5% CO_2 at 37°C. Each Warburg flask contained 2 to 3×10^5 cells in 3 ml reaction volume, with 55 μmoles of glucose and 32 μmoles of freshly neutralized cysteine hydrochloride as substrate. Each test was run for 60 minutes. The number of amebae used throughout these experiments produced 20-24 μmoles CO_2 and 4-6 μmoles of H_2S in 60 minutes under standard conditions. With each experiment controls containing no inhibitor were set up. The activities of controls were taken as 100. Both rates of CO_2 and H_2S formation measured in the presence of the inhibitors are expressed (in the Table) as the per cent activities of the controls.

The following compounds were synthesized in this laboratory: isonitrosoacet-p-aminobenzoic acid(7), 6-bromo-2-nitrobenzoic acid(8), pyocyanine(9), 2, 3-diaminophenazine(10, 11), and 2-amino-3-hydroxyphenazine(10). A mixture of 20,000 units streptomycin sulfate and 20,000 units of penicillin G per flask is abbreviated as "antibiotics" in the Table. The compound called 242-L is a chelating agent for heavy metals (Alrose Chemical Co., Providence, R. I.,), the composition of which was undisclosed.

Results. The effect of inhibitors is combined in the table, which also shows the substrates used in each experiment. A mixture of streptomycin and penicillin, which was previously found to arrest the growth of the bacteria, associated with *E. histolytica* in cultures, had an inhibitory effect on the metabolism of the protozoon itself. This effect was an immediate one, and could therefore not be explained by an indirect action. The CO_2 and H_2S evolution are specific reactions of the protozoon(1-3) and do not occur in suspensions of associated bacteria. This observation also indicates that the antibiotics act on enzyme systems of *E. histolytica*. The magnitude of inhibition depends on the substrates used. The H_2S evolution is inhibited 90-96% when glucose and cystine or cysteine alone are the substrates. The direct, and probably reversible action of these antibiotics on *E. histolytica* may explain their transient beneficial effect in amebiasis, which was previously attributed to an indirect action through suppression of bacterial growth(12).

The inhibitors from No. 2 to 8 are known to act by their metal complex forming properties. Both CO_2 and H_2S evolution are suppressed, suggesting that a metalloprotein plays an important role in the metabolic system of *E. histolytica*. The inhibitory effects of aminobenzoates run parallel with their ability to form metal complexes, orthoaminobenzoate being the more effective inhibitor of H_2S formation.

Metadinitrophenol affects CO_2 evolution to a greater extent than H_2S production, so does pyocyanine. The latter may act by virtue of its reactivity with –SH enzymes. A variety of –SH reagents (not listed here) also inhibit CO_2 evolution. The synthetic substances: 6-bromo-2-nitrobenzoate and 2,3-diaminophenazine are of particular interest. Both of these compounds, particularly 2,3-diaminophenazine, activate H_2S evolution but only when both glucose and cysteine are the substrates. Various dyes (No. 17 and 18) and arsenate (No. 5) act primarily on CO_2 formation from glucose. Methylene blue and arsenate have no effect on H_2S evolution at all; they both suppress the decarboxylation of pyruvate.

The inhibitory effect of fumarate and maleate can be explained by the reactivity of the ethylenic bond in these molecules with thiols. Maleate reacts readily with cysteine, to form 2-amino - 2-carboxymethyl - mercaptosuccinic acid(13), and was also found to be a more powerful inhibitor than fumarate. Neither of them inhibit pyruvate decarboxylation, therefore the suppression of CO_2 evolution (by maleate) from glucose must be explained by an interference with the thiol system. Exposure to 100% O_2 for 10 to 30 minutes causes an irreversible inactivation of these enzyme systems.

Discussion. The results of previous studies, dealing with the pathway of anaerobic oxidation in *E. histolytica*(1,2,3), enabled us to investigate the mechanism of action of certain metabolic inhibitors. It may become now fruitful to search for common points of attack of generally used amebicides as summarized by Balamuth(14). It is suggested that drugs may be classified on the basis of model experiments, as here described. Certain inhibitors (antibiotics) interfere primarily with enzyme systems which catalyze the reduction of sulfur; other drugs may combine with a metal component of the enzyme system of *E. histolytica*. The most potent inhibitors were found among certain phenazine and thiazine derivatives. Substituted phenazines combine their metal binding properties with their ability to react with thiol radicals. For instance, we obtained evidence which indicates that a labile S containing phenazine derivative was formed when 2,3-diaminophenazine was incubated under anaerobic conditions with a suspension of actively metabolizing *E. histolytica*. The isolation of this substance (characterized by marked color changes) failed however, since it decomposed to H_2S and 2,3-diaminophenazine after extraction, as soon as exposed to air. It is suggested that new chemotherapeutic agents which combine certain properties of the type of inhibitors described above may prove to be more selective amebicides, since they interfere with specific enzyme systems of the protozoon.

Summary. The *in vitro* effects of several groups of compounds were tested on the rate of CO_2 and H_2S evolution, catalyzed by enzyme systems of *E. histolytica*. Since the mechanism of the enzymatic reduction of S to H_2S, a reaction which is coupled with the anaerobic oxidation of sugars in this protozoon, has been previously elucidated, it was possible to classify inhibitors on the basis of their primary point of attack. Certain anti-

biotics were found to interfere with the S reducing enzyme system, while compounds which form metal ion complexes inhibit both CO_2 and H_2S evolution to an equal extent. Arsenate and methylene blue on the other hand inhibit primarily CO_2 evolution (the decarboxylation of pyruvate). Certain heterocyclic N containing compounds (*e.g.*, 2, 3-diaminophenazine), which can trap HS° and also form complexes with metal ions were found to be the most potent inhibitors of enzyme systems of *E. histolytica.* The metabolic reactions which were investigated are specific for this protozoon, therefore inhibitor studies on these enzyme systems are considered to be the biochemical basis of chemotherapy of amebiasis.

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Complement Activity in the Eviscerate Rat.* (21890)

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Depression of complement activity occurs in guinea pigs in which liver damage has been induced by chloroform, ethionine(1) or carbon tetrachloride(2). This report concerns observations on the functionally eviscerate rat in which complement activity falls to 5-10% of normal values by 24 hours.

Methods. Functional evisceration under sodium cyclopal anesthesia was performed on Sprague-Dawley rats weighing 300-375 g. The operation was single stage(3). Sham operated animals were maintained by multiple small subcutaneous injections of sodium phenobarbital. All animals were placed in a chamber at 26° ± 1° C and given 15% dextrose with streptomycin and penicillin by constant intravenous infusion at the rate of 20 cc/rat/24 hours. At 8, 14, 16, or 24 hours bleedings were made from the abdominal aorta. The blood remained at room temperature for 1½ hours. Rimming was carried out and all samples were placed in the ice box. At 16 hours they were centrifuged at 1000 rpm for 35 minutes at room temperature. The supernatant was siphoned off and stored at -20°C. Within 72 hours complement titrations were performed either by the 100% end point visual method(4) or by the 50% hemolysis end point determined photometrically(4). Normal and eviscerate bloods, or eviscerate and sham bloods were always titrated simultaneously.

It has been reported(2) that complements

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TABLE I. Level of Complement Activity in Blood of Eviscerate Rat.

End point used	Delayed titration				Immediate titration	
	Complement 100% units per ml		Complement 50% units per ml		Complement 50% units per ml	
Group	N	Avg ± S.E.	N	Avg ± S.E.	N	Avg ± S.E.
Normal controls	6	32 ± 3.6	8	202 ± 22.6	9	209 ± 12.9
Eviscer. 8 hr			6	75 ± 12.3	6	102 ± 11.1
Idem 14 hr	4	5 ± 1.9				
Idem 16 hr			8	18 ± 2.8	6	46 ± 12.8
Idem 24 hr	10	1 ± .14	13	5 ± .87	9	16 ± 2.0
Shams 14 hr	2	32 ± .0				
Idem 24 hr	10	30 ± 4.1				

low especially in C′3 and C′4 obtained from guinea pigs treated with carbon tetrachloride were often so unstable that overnight storage in the refrigerator resulted in disappearance of all remaining hemolytic activity. In order to determine whether *in vitro* degeneration was participating in the complement drop, other normal and eviscerate animals were bled and their complements titrated immediately.

For the titrations a 2% rbc suspension was made from fresh blood of the same sheep and photometrically standardized. Veronal-saline buffer with Mg and Ca added(5) was used as the diluent and in other particulars our scheme was according to Kabat and Mayer (4). On separate occasions 3 pools of 24-hour eviscerate sera containing 3 samples each and 1 pool of 8-hour eviscerate sera containing 4 samples were tested for anticomplementary activity. Comparison was made between heated normal and heated test sera using a 50% end point. Serum providing complement was diluted in series; the concentration of the serum tested for anticomplementary activity was kept constant.

Results. The findings (Table I) by the two methods are essentially the same and depict a depression of complement which is marked by 16 hours and more profound by 24 hours. *In vitro* degeneration occurring between the time of bleeding and titration is responsible for only a minor portion of this complement depression. The complement titers of sham operated rats remain essentially unchanged (Table I). In eviscerate rat sera no anticomplementary activity could be demonstrated.

Discussion. Complement depression must be due to its catabolism and failure of synthesis. It is not due to development of seral anticomplementary activity after evisceration. Dick(6) proposed the liver as the source of serum complement. We suggest that inactivation of the liver may be principally responsible for the depression of complement in the blood of the eviscerate rat.

Summary. In the eviscerate rat depression of complement titer is progressive, reaching 5-10% of normal values by 24 hours.

We wish to express our appreciation to Drs. M. H. Kuizenga and H. F. Hailman of the Upjohn Co. who supplied the cyclopal, streptomycin and penicillin.

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Production of Arteriosclerosis in Birds by the Prolonged Feeding of Dihydrocholesterol.* (21891)

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It was demonstrated recently, both in birds and rabbits, that the rise in plasma cholesterol and the incidence of arteriosclerosis resulting from the feeding of diets rich in cholesterol can be greatly reduced by the addition of dihydrocholesterol (DHC) to those diets(1,2). In the experiments with birds, the feeding periods lasted 11 weeks and in those with rabbits, 9 weeks. In the same experiments, it was also observed that 2 of 8 rabbits that were fed a diet containing 2% DHC developed some arteriosclerosis. This finding led us to undertake a study of the effects of the protracted feeding of DHC. The present report deals with the histological changes observed in the aortas and livers of birds that had been fed a diet containing 0.5% DHC for 6 months.

Experimental. The birds used in this study were White Leghorn cockerels, maintained up to the age of 5 months on a Purina Broiler Chow. After that period, 12 birds were fed Purina Broiler Chow to which had been added 0.5% DHC† and 2.5% cottonseed oil. Twelve other birds, which served as controls, received the Purina Broiler Chow to which had been added 2.5% cottonseed oil. The feeding of these 2 diets was continued for 6 months. At the end of that time, all birds were sacrificed by exsanguination. The thoracic and abdominal aortas were removed and opened longitudinally, and intimal lesions were noted and graded. The aortas were then fixed in neutral formalin, sectioned, and graded as described previously(3,4). The aortic sections were stained with hematoxylin and eosin, Sudan IV and hematoxylin, and Nile blue. They were also stained by the Laidlaw (reticulum), Weigert-Van Giesen (elastic tissue, collagen), and Schultz methods (cholesterol)(5). Unstained, frozen sections were examined with polarized light. The livers were excised and weighed, and sections were prepared for microscopic examination. The hepatic sections were stained with hematoxylin and eosin, Sudan IV and hematoxylin, and Nile blue, and also by the Schultz method (5). Unstained, frozen sections were examined with polarized light.

Gross examination of tissues. DHC-Fed Birds. Thoracic Aorta. All but 2 of the DHC-fed birds showed intimal plaques in their thoracic aortas. These plaques were oval, yellow, elevated, often multiple and coalescing, and displayed wrinkled surfaces. The areas of these plaques ranged from 3 x 1 mm to 28 x 10 mm. *Abdominal Aorta.* All DHC-fed birds had one or more elongated, longitudinal, ridge-like plaques in their abdominal aortas, and in almost all instances these plaques were distinctly yellow. These plaques varied from 4 to 38 mm in length and from 1 to 6 mm in width. Considerable transverse ridging and wrinkling of the abdominal aortic intima were also observed.

Liver. There was a pronounced increase in the size of the livers of birds fed DHC. The average liver weight for the 12 DHC-treated birds was 77.8 ± 20‡ g, whereas the average hepatic weight of the 8 control birds was 26.2 ± 3 g. The livers of the DHC-fed birds were yellow-tan in color, and their parenchymas were exceedingly granular and friable (Fig. 1).

Control Birds. Thoracic Aorta. Only 2 of the control birds had non-elevated, yellow intimal lesions of the thoracic aorta. In one bird the lesions were coalescing, and the total area of the lesion measured 17 x 5 mm. *Abdominal Aorta.* All but one of the control

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* Aided by grants from the United States Public Health Service and the Life Insurance Medical Research Fund.

† We are indebted to the Schering Corporation for the dihydrocholesterol used in this study.

‡ Average deviation from the mean.

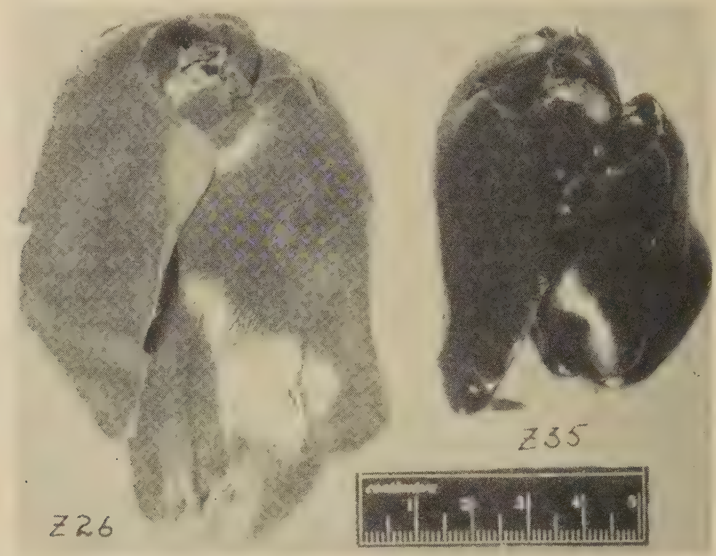

FIG. 1. Livers of DHC-fed and control birds. Note enlargement and pallor of liver of DHC-fed bird on left (reproduced from Kodachrome × ¾).

birds had longitudinal, ridge-like, intimal plaques of the abdominal aorta, but these were fewer and smaller than those observed in the DHC-fed birds and as a rule were grey or white in color. These plaques were usually less than 17 mm. in length.

Microscopic examination of tissues. DHC-Fed Birds. Thoracic Aorta. All birds showed varying degrees of diffuse intimal thickening of their thoracic aortas, and this thickening consisted mainly of enlarged fibroblasts and fewer macrophages with abundant and vacuolated cytoplasm. The overlying endothelial cells were likewise swollen and vacuolated. A rich reticulum surrounded the individual intimal cells, and in the larger lesions delicate, collagenous fibers had also appeared. In the thickest portion of the intima, the deeper layers were relatively acellular and the collagenous stroma was more abundant, condensed, and hyalinized. The intimal cells appeared to originate from the endothelium. In the earlier lesions the intimal cells had an irregular arrangement that tended to become perpendicular as the lesions enlarged. Considerable vacuolization of the connective tissue and smooth muscular cells of the inner third of the media was also noted. Sudan IV staining showed abundant lipid droplets in the vacuolated intimal cells and in the swollen cells in the inner third of the medial layer (Fig. 2). This lipid substance stained violet to dark blue with Nile blue. In the intimal layer of only one bird a small amount of refractile substance was visible with polarized light. As judged by the Schultz reaction, cholesterol was absent in the thoracic aortas of the DHC-fed birds. *Abdominal Aorta.* All the birds of this group showed considerable diffuse intimal thickening of the abdominal aorta, usually with the formation of large intimal plaques. The thickened intima consisted of vacuolated fibroblasts and macrophages. In the earlier lesions these cells had an irregular arrangement, but as the thickening became more pronounced, the cells assumed a circumferential arrangement. In the larger plaques the superficial and deeper portions were more compact and cellular, whereas the central portions were relatively acellular, appeared mucoid, and often contained empty, cleft-like spaces resembling those produced by cholesterol deposition.

Rich reticulum and collagenous fibers were found in the thickened intima and intimal plaques. This fibrillary material tended to be condensed in the superficial and deeper portions of the larger plaques; considerably less was found in the central or deeper mucoid portions. Beneath the thickened intima, and

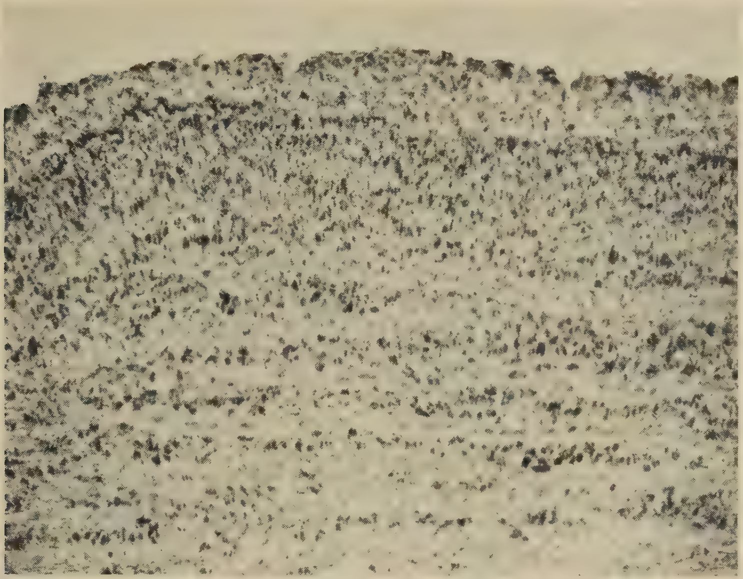

FIG. 2. Thoracic aorta of DHC-fed bird (No. Z22). The intima is thickened, and both intimal and medial layers are infiltrated with lipid. (Sudan IV and hematoxylin. × 125.)

particularly beneath the larger plaques, the internal elastic membrane was flattened, atropic, and fragmented. It frequently showed deficient segments. Newly formed elastic tissue did not appear in the intimal plaques. Beneath some of the largest intimal lesions the media was compressed and sometimes absent. Groups of foamy macrophages and fibroblasts occasionally penetrated the defective internal elastic membrane and extended into the medial layer.

Sudan IV staining showed large amounts of lipid material appearing as fine and coarse droplets throughout the thickened intima and intimal plaques, and most abundant within the numerous macrophages (Fig. 3). This substance stained violet to dark blue with Nile blue. Examination with polarized light showed considerable amounts of refractile crystalline material in the thickened intima and intimal plaques. These crystals were elongated and spindle-shaped, and appeared larger than cholesterol crystals. None of the lipid substance gave a positive reaction with

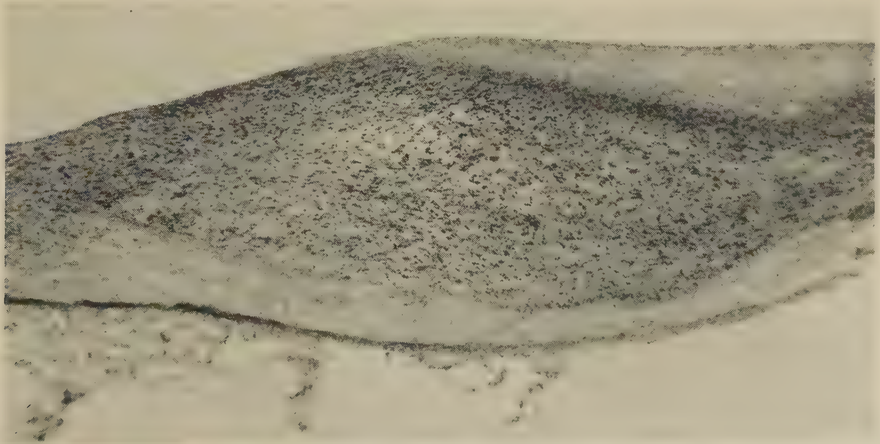

FIG. 3. Abdominal aorta of DHC-fed bird (No. Z22). The large intimal plaque contains large amounts of lipid. (Sudan IV and hematoxylin. × 40.)

the Schultz test. However, in several of the larger plaques, the intercellular substance between the foam cells did give a very faintly positive Schultz reaction.

Liver. The livers of all the birds in this group showed extensive infiltration of the sinusoids by groups of swollen, vacuolated macrophages which crowded, displaced, and distorted the adjacent hepatic parenchymal cells. The foam cells showed no particular localization within the hepatic lobules. These cells contained oval or round nuclei that had delicate chromatin and one to two small nucleoli. The cytoplasm was granular and vacuolated. In these cellular collections, moderate numbers of lymphocytes and more numerous eosinophilic leukocytes were present. Fibrosis had not occurred. Sudan IV staining showed moderate amounts of lipid material in fine and coarse droplets localized mainly in the sinusoidal macrophages. Smaller amounts were found in scattered hepatic parenchymal cells. This substance stained violet to dark blue with Nile blue. Examination with polarized light showed refractile crystalline material in the clumped macrophages in four of the birds. The Schultz reaction on the hepatic tissues was negative.

Control Birds. Thoracic Aorta — A few very small areas of intimal thickening were observed in the thoracic aortas of only 4 of the birds. These areas consisted of swollen, vacuolated endothelial cells and a small amount of delicate fibrillary material. Sudan IV staining showed small collections of fine lipid droplets in the inner third of the media and in the minimally thickened intima in four of the birds. The substance did not stain with Nile blue. No refractile material was demonstrable with polarized light. The Schultz reaction was negative.

Abdominal Aorta. All control birds showed intimal lesions of the abdominal aorta either in the form of small, localized plaques or as more diffuse but mild intimal thickening. The intimal lesions consisted of fibroblasts arranged irregularly in the smaller lesions and circumferentially in the larger plaques. Coarse reticulum fibers and delicate collagenous fibers enveloped the intimal connective tissue cells. Beneath the intimal lesions the internal elastic membrane was invariably fragmented, frayed, but rarely reduplicated. No elastic tissue was found in the intimal lesions. Small amounts of lipid material appearing as fine droplets in the cytoplasm of the connective tissue cells were observed in all but one of the birds. This lipid was localized in the deeper portions of the thickened intima or intimal plaques. This substance stained violet with Nile blue, was not demonstrable as refractile material with polarized light, but did give a distinctly positive Schultz reaction.

Liver. The hepatic tissues of the control birds were entirely normal. Sudan IV staining showed a few scattered lipid droplets in some hepatic cells in two of the 8 birds. This substance neither stained with Nile blue nor gave a positive Schultz reaction.

Comment. It is shown here that the feeding of DHC for long periods of time induces extensive arteriosclerosis of the thoracic and abdominal aortas. The aortic and hepatic lesions observed in the DHC-fed birds were identical with those resulting from the feeding of cholesterol(3,4,6). Lipid infiltration of both the thoracic and abdominal aortas led to intimal fibrosis and plaque formation which were particularly severe in the abdominal aortas. The crystals found in the aortic lesions appeared larger than did cholesterol crystals seen in the aortic walls of birds fed cholesterol. This crystalline substance reacted negatively to the Schultz test, and presumably was dihydrocholesterol. Cholesterol-reacting material demonstrable by the Schultz method was present, however, in the abdominal aortic lesions of the control birds.

Hepatic enlargement in the DHC-fed birds apparently resulted from lipid (partly dihydrocholesterol) infiltration into the sinusoidal reticuloendothelium, and led to distortion of the parenchymal cells. Gould(7) has recently shown that, in rats fed DHC for 5 days, partial replacement of cholesterol by DHC occurs in the liver.

Summary. 1. The feeding of a diet containing 0.5% dihydrocholesterol (DHC) for 6 months resulted in the development of severe arteriosclerosis of the thoracic and abdominal aortas in 12 birds. 2. The aortic

lesions observed were identical with those induced in the bird by the feeding of cholesterol, and appeared to have resulted from deposition of dihydrocholesterol in the vascular wall. 3. Hepatic enlargement in DHC-fed birds was similar to that observed previously in cholesterol-fed birds, and was due in part to reticuloendothelial storage of dihydrocholesterol.

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Cardiac Lesions in Rabbits After Pharyngeal Infections with Group A Streptococci.* (21892)

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The concept of a relationship between antecedent group A hemolytic streptococcal infection and rheumatic fever is supported by an impressive body of data(1), but the pathogenesis of rheumatic fever remains an unsolved problem, and many investigations have been directed toward the development of a suitable experimental model of the disease(2). In previously reported studies from several laboratories, cardiac lesions resembling those of human rheumatic fever have been described in animals following skin infections with group A streptococci(3,4), and we have produced similar lesions in rats after repeated induction of streptococcal pneumonia(5). However, the considerations (a) that streptococcal infections in man characteristically involve the pharynx(6), and (b) that the site of infection may be important in the genesis of rheumatic fever(7), led us to attempt to design a method whereby pharyngeal infections in rabbits could be produced with group A streptococci. The successful development of such a method(8) has made it possible to simulate in rabbits conditions which in man may be associated with the occurrence of rheumatic fever, and it is the purpose of this communication to describe in a preliminary manner the cardiac changes observed when this technic was employed.

Methods. Animals. New Zealand rabbits from a single source, weighing 3 to 5 kg and free of demonstrable infection, were used. The animals were fed Purina rabbit pellets and were given water *ad libitum.*

Cultures. The following strains of group A streptococci were employed: IGL-23 (type 17), S23 Burbank (type 14), C203 (type 14), 41439 (type 3) and AF-1 (type 13). As detailed previously, suitable dilutions of 4-hour cultures were made so that the inocula contained from 2 to 9 x 10^6 chains(8).

Induction of pharyngeal infections. The technic employed was identical with that already described(8) except that a more effective head restraint obviated the need for ether inhalation as a supplementary anesthetic, and most of the animals, therefore, received only 1.0 ml of pentobarbital sodium intraperitoneally. A total of 35 rabbits was subjected to

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* Supported by a grant from the Life Insurance Medical Research Fund.

† Student Research Fellow when this work was done.

streptococcal infection; 29 were infected once and sacrificed at given intervals thereafter, whereas the remaining 6 underwent multiple inoculations. Although anti-microbial therapy was not used, all blood cultures taken at the time the animals were sacrificed were negative; transient bacteremia occurred occasionally in the first 24 hours after inoculation.

Controls. Three groups of animals were included: (a) *Sham controls.* Twelve rabbits were subjected to the identical procedures used for induction of streptococcal infection except that a sterile broth inoculum was used instead of bacterial culture. Eight of the animals were sacrificed after one such exposure, and the remainder after 3; (b) *Comparative controls.* This group was made up of 43 rabbits, used for unrelated acute experiments, in most of which death followed the injection of sterile plastic beads. None of the animals was given bacteria or bacterial products; (c) *Pneumococcal controls.* Seven rabbits were infected intrapharyngeally with virulent type III pneumococci; the inoculum contained a comparable number of bacteria to that employed in the streptococcal studies. The animals were sacrificed 72 hours after inoculation. *Histopathologic Studies.* At the conclusion of the period of observation, the animals were sacrificed by injection of air or of lethal doses of pentobarbital sodium into the ear veins. Autopsies were done promptly and representative sections of the atria, ventricles and valves were taken for microscopic examination. The blocks were fixed in 10% Zenker-formalin; hematoxylin and eosin as well as a number of other stains were used.

Results. Streptococcal series. Initially, 8 rabbits were infected intrapharyngeally. Two animals died 8 and 16 days respectively after induction of infection; the remainder were reinfected with various strains of group A streptococci 3 to 6 times, at intervals ranging from 3 weeks to several months. Seven of the 8 rabbits in this group, including the 2 that died after single infections, exhibited striking lesions in the myocardium, characterized by muscle necrosis, disruption of collagen, replacement fibrosis and infiltration with histiocytes, lymphocytes, Anitschkow myocytes and occasional multinucleated giant cells.

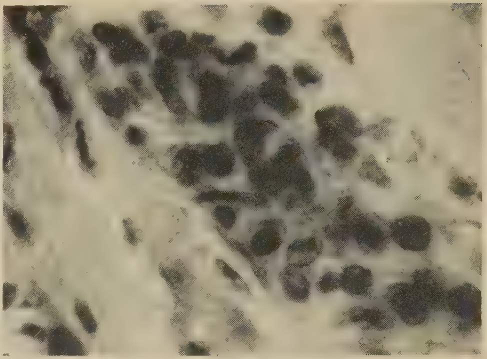

FIG. 1. Myocardial necrosis with cellular infiltration in a rabbit sacrificed 3 days after a single pharyngeal infection with group A streptococci. (H & E × 666.)

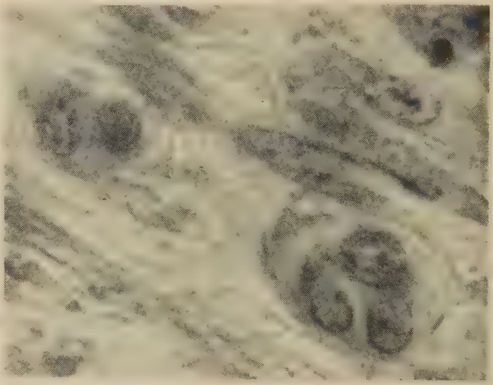

FIG. 2. Multinucleated giant cells, similar to those seen in lesions of human rheumatic carditis, in an endocardial lesion from a rabbit subjected to multiple pharyngeal infections with group A streptococci. (PTAH × 1500.)

The lesions bore no constant relationship to blood vessels, although small arteries were observed in some of the areas of necrotic myocardium. The adventitia of such vessels was infiltrated with inflammatory cells but the other coats were not involved. Some foci of myocardial necrosis extended to the endocardial surface, and a few of these lesions contained large numbers of multinucleated giant cells of the type seen in human rheumatic fever (Fig. 1 and 2). No valvular changes were observed. The pathology and pathogenesis of the lesions will be described and illustrated in detail in a subsequent communication(13).

Careful search of suitably stained sections for viral inclusion bodies, bacteria and parasites was made but none was found. Like-

TABLE I. Occurrence of Myocardial Necrosis and Cellular Infiltration in Control Animals.

Control group	Positive	Negative
Sham	0	12
Comparative	1	42
Pneumococcal	0	7
Total	1	61

wise, cultures of ground myocardium were all negative.

Control animals. No cardiac lesions of the type observed in the 35 rabbits subjected to streptococcal infection were seen in any of the 19 animals comprising the sham and pneumococcal control groups. Similarly, the hearts of 42 of the 43 rabbits in the comparative control series were likewise free of myocardial necrosis and cellular infiltration such as described in the rabbits infected with streptococci; the 1 remaining animal had only a single small area of necrotic muscle (Table I). The hearts of most rabbits in all the groups contained small foci of lymphocytes and monocytes, without associated muscle necrosis; it has been demonstrated that such foci occur spontaneously in this species(9).

In view of the fact that 7 out of 8 rabbits subjected to streptococcal pharyngeal infections, including 2 that had only a single infection, exhibited significant myocardial lesions, it was decided to submit additional rabbits to a single pharyngeal inoculation in order (a) to confirm the very high incidence of positive results, and (b) to determine how early lesions could be demonstrated. Accordingly, 27 animals were infected as before, and groups were sacrificed respectively 1, 2, 3, and 7 days later. Focal, necrotizing myocardial lesions comparable to those observed in the first experimental series were encountered in 6 of 8 rabbits sacrificed 2 days after infection, in all the animals killed 3 days after inoculation and in 5 of 6 sacrificed on the 7th day after inoculation (Table II). Fibrosis was, of course, absent or minimal in these lesions of short duration but the inflammatory cell infiltrate was similar.

TABLE II. Occurrence of Myocardial Necrosis and Cellular Infiltration After Streptococcal Pharyngeal Infection.

Days after infection	Positive	Negative
1	3	1
2	6	2
3	9	0
7	5	1
Total	23	4

In general the myocardium was less extensively involved in the "single infection" rabbits than in the "multiple infection" group. As already indicated, repeated attempts, by means of appropriate technics, to demonstrate bacteria, were unsuccessful, and granulocytes were only rarely seen, even in the most acute lesions.

Discussion. The observations recorded herewith are significant as follows: 1). They indicate that in the rabbit pharyngeal infections with group A streptococci are followed in almost every instance by the appearance of a type of carditis rarely seen in control animals and not due to direct bacterial invasion. The experimental conditions bear similarity to those that, in man, may be associated with the development of rheumatic fever. 2). The lesions in the myocardium are characterized by muscle necrosis, cellular infiltration with histiocytes, lymphocytes, multinucleated giant cells, and Anitschkow myocytes and by the disruption of collagen and development of fibrosis. These changes, and particularly the cytologic features, are also seen in rheumatic fever. Despite the fact that the configuration of the lesions was not such as to warrant their identification as Aschoff bodies, this single difference may not be of basic importance, and may be attributable to variation in species reactivity. 3). The occurrence of myocardial lesions within 24 hours of induction of a single infection indicates that their pathogenesis involves a non-immunologic mechanism, perhaps a proteolytic substance such as was employed by Kellner and Robertson(10,-11), and raises the question as to whether human rheumatic fever is a disease due solely to an inappropriate antigen-antibody reaction (12). 4). The extent of the damage to the myocardium encountered in some of the repeatedly infected rabbits suggests that eventually cumulative damage occurs; conceivably a degree of involvement might be reached at which compromise of cardiac function will ensue.

Studies now in progress(13) should permit more detailed definition of the nature and pathogenesis of the lesions produced and of their possible relation to those of human rheumatic fever.

Summary. The majority of rabbits subjected to single intrapharyngeal infections with group A streptococci developed cardiac lesions characterized by muscle necrosis, cellular infiltration with mononuclear cells and Anitschkow myocytes and disruption of collagen. After multiple infections, fibrosis occurred and giant cells were also observed. Bacteria could not be demonstrated in any lesion. In 3 control groups, comparable lesions were absent in the hearts of all but one rabbit. The appearance of myocardial lesions within 24 hours of induction of pharyngeal infection suggests that a non-immunologic mechanism is involved.

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Effect of Various Doses of Total Body X-Irradiation on Total Amount of Deoxyribonuclease II in Rat Spleen.* (21893)

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It was reported from this laboratory(1) that total-body x-irradiation caused an increase in activity of the deoxyribonuclease (DNase) of spleen whose pH optimum is close to 5 (DNase II(2)). The present paper reports a study of the change in specific activity of the enzyme with various doses of total body x-irradiation and correlates this change with the change in spleen weight-body weight ratio.

Experimental. Female Sprague-Dawley rats weighing between 80 and 150 g were used. The animals were fed a diet of Purina laboratory chow and were given food and water *ad libitum*. The irradiations were carried out using the following physical factors: 220 KV, 15 ma, inherent filtration only, HVL 0.25 mm Cu, 32 inches to the center of the animal and at a rate of 35.5 r per minute in air. The animals were weighed and killed by stunning and decapitation 24 hours after irradiation. The spleens were quickly removed, weighed, and homogenized in an all-glass homogenizer using cold distilled water as the suspending medium. Dilutions were made to give approximately 20% homogenates. The enzyme was assayed by incubating 1 ml of homogenate with 1 ml of a solution of highly polymerized deoxyribonucleic acid (DNA)† containing 5 mg per ml in 0.1 M acetate buffer, pH 5.0, for 1 hour at 37°. At the end of

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† Purchased from California Foundation for Biochemical Research, Los Angeles.

TABLE I. Effect of Various Doses of X-Irradiation on Spleen Weight-Body Weight Ratio, Specific Activity of Spleen DNase II, and Total Activity of Spleen DNase II.

Dose	$\frac{\text{Spleen wt}}{\text{Body wt}} \times 10^3$	Specific activity of spleen DNase II*	Total activity of spleen DNase II†
Control (14)	3.55 ± .18‡	9.01 ± .60	31.99 ± 1.70
50 r (5)	2.15 ± .11	14.88 ± .56	31.76 ± .52
100 r (9)	2.26 ± .12	14.69 ± .76	33.20 ± 1.85
200 r (6)	1.94 ± .14	16.95 ± .54	32.71 ± 2.14
400 r (4)	1.83 ± .08	19.35 ± 1.44	35.41 ± 3.07
500 r (6)	1.72 ± .14	20.19 ± 1.14	34.73 ± 2.93

* Expressed as μg DNA depolymerized/mg wet tissue/hr.
† " " μg DNA depolymerized/spleen/g body wt/hr.
‡ Standard error.
Numbers in parentheses refer to No. of animals.

the incubation period, 2 ml of ice-cold 10% trichloroacetic acid (TCA) were added, the solutions centrifuged, and filtered. Tissue blanks were prepared by incubating the homogenate for 1 hour in the absence of added substrate and, at the end of the incubation, adding the substrate and quickly adding the TCA. Two milliliter aliquots of the filtrates were subjected to the Dische reaction(3) and the violet color determined in a Klett-Summerson photoelectric colorimeter using the 59 filter. The quantity of DNA depolymerized was then calculated from a standard curve. A reagent blank containing the diphenylamine reagent and distilled water was used to adjust the colorimeter to full transmission.

Results and discussion. Table I shows that while the specific activity of the enzyme increased with increasing doses of x-irradiation up to 400-500 r, the total activity per spleen was found to be essentially unchanged. The mechanism for the increased activity on irradiation therefore appears to be a selective retention of the enzyme by the spleen at least 24 hours post-irradiation. Similar mechanisms for the increased activity of other spleen enzymes have been reported(4-6). It is of interest to note that Ashwell and Hickman(4) found that the specific activities of succinic dehydrogenase and cytochrome oxidase were unchanged despite the splenic involution accompanying total-body irradiation. In the previous paper from this laboratory(1) it was reported that the DNase whose pH optimum is close to neutrality does not show increased specific activity in spleen following 500 r of total-body irradiation.

Summary. The increases in activity of spleen DNase II following a series of different doses of total-body x-irradiation 24 hours after treatment have been measured. These increases have been correlated with the change in spleen weights associated with the irradiation.

The authors wish to express their gratitude to Doctor I. Meschan, Professor of Radiology for his cooperation in irradiating the animals, and to Mr. George Pipkin for his technical assistance.

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Metabolism of Testosterone by Homogenates of Ovarian Adenocarcinoma.[*]
(21894)

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Testosterone is metabolized by homogenates of a number of tissues of experimental animals(1). The availability[†] of an ovarian adenocarcinoma from a patient made it of interest to determine whether such a tissue also could metabolize testosterone.

Procedure. The ovarian adenocarcinoma, removed surgically from an 80-year-old female, weighed 76 g, was yellow-white in appearance, free of extraneous fat and contained practically no blood. The tumor was divided (Table I) within 30 minutes of removal and homogenized for 20 seconds in a Waring Blendor with 50 ml of 0.166 M sodium pyrophosphate buffer (pH 9.0). The homogenates were transferred to Fernbach flasks, the Waring Blendors washed with another 50 ml of buffer, the additives introduced and the mixture incubated with shaking in a water bath at 37.5° for 1.5 hours. The incubation was terminated by the addition of 750 ml of ethyl alcohol, filtered, the alcohol extract concentrated *in vacuo* at 40° then extracted with ether (4 x 800 ml) and the phospholipids removed by acetone precipitation. The resulting lipids were submitted to alumina column chromatography with the following solvent sequence: carbon tetrachloride, benzene, ether, and methyl alcohol. The fractions were pooled according to their elution properties. These fractions were then submitted to paper chromatography(2). The androstenedione was assayed by absorption at 241 mμ in a Beckman DU Spectrophotometer and a modified Zimmermann color reaction(3). The testosterone recovered was determined by absorption at 241 mμ.

Results. The only endogenous steroid isolated was cholesterol (Table I). It occurred in a constant amount and apparently was not affected by the incubation procedure. It was identified by melting point, mixed melting point and its infra-red spectrum. Endogenous estrogens as well as androgens were not evident either before or after incubation. (Table I, Exp. 3 and 4).

The homogenate was able to effect a slight conversion of testosterone to Δ^4-androstene-3, 17-dione in the presence of nicotinamide. The metabolite was indicated by its behavior on the paper chromatogram, ultraviolet absorption and color reactions. The addition of diphosphopyridine nucleotide (Pabst) enhanced the effectiveness of the tumor tissue. The amount of conversion was relatively small but was sufficient to provide further characterization by its infra-red spectrum.

Discussion. Ovarian adenocarcinoma of human origin possesses a small amount of apoenzyme necessary for the metabolism of the 17β hydroxyl group of testosterone to the 17 keto group. The amount of this enzyme on

TABLE I. Metabolism of Testosterone by Homogenate of Human Ovarian Adenocarcinoma.

					Steroids isolated		
Exp. No.*	Tissue, g	Testosterone, mg	Nicotinamide, mg	DPN, mg	Cholesterol, mg	Androstenedione, mg	Testosterone recovered, mg
1	12	50	400	—	21	.08	41.2
2	12	50	400	50	32	1.1	41.7
3	12	—	—	—	29		
4	40	—	—	—	100		

* In Exp. 1, 2, and 3 the tissue was homogenized with 50 ml 0.166 M sodium pyrophosphate (pH 9.0), transferred to Fernbach flasks with 50 ml buffer and incubated with shaking for 1.5 hr at 37°. In experiment 4 tissue was homogenized immediately with 1 liter ethyl alcohol.

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[†] We are indebted to Dr. Byron Bailey for securing this tissue.

a unit tissue basis is roughly only 1/30th that observed in homogenate of liver of guinea pigs (unpublished) studied in the same manner. It is of interest that not even trace amounts of endogenous androgens or estrogens could be detected.

Summary. Ovarian adenocarcinoma (human) contains a small amount of the apoenzyme necessary for the oxidation of the 17β hydroxyl group of testosterone. Endogenous estrogens or androgens could not be detected but cholesterol was present at 212 mg/100 g of tumor.

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Effect of Limitation of Protein Intake on Hepatic Uricase and on Urinary Uric Acid and Allantoin.* (21895)

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It has been suggested that the change in the urinary uric acid-allantoin ratio in dogs bearing hepatic tumors is a reflection of decreased uricase activity(1). If this relationship could be demonstrated it would provide a means for determining the activity of an enzyme system in the intact animal. Since preliminary experiments had indicated that protein deprivation lowered the uricase content of rat liver, it was decided to determine the effect of protein deprivation on the uric acid and allantoin content of the urine.

Experimental. Fifteen male rats obtained from the Wistar Institute were used. The animals were placed in isolation for one week maintained during that period on Purina fox chow and injected once with 60,000 units of Penicillin-G in oil. They were then placed in individual metabolism cages and fed the basal diet containing 18% casein. At the beginning of the experiment the animals were divided into 3 groups of equal weight. Five rats (Group I) were fed a protein-free diet (isocaloric substitution of dextrose). Five rats (Group II) were placed on the 18% casein diet and pair-fed with Group I. Five rats (Group III) were maintained on the 18% casein diet *ad libitum*. Details of diet and treatment of these animals have been described by Wainio *et al.*(2). The animals used were those of Experiment 1 of that publication.

During the first 3 days of each week urine was collected twice daily and the cages rinsed with distilled water. Care was taken to exclude food and fecal material from the urine. The urines and washings were stored in the deep freeze. At the end of each 3-day period the pooled samples were thawed and diluted to volume for allantoin and uric acid determinations.

At the end of 7 weeks the animals were sacrificed by decapitation. The livers were removed and homogenized with 3 volumes of water.

Methods. Uricase activity was determined manometrically by a modification of Holmberg's method(3). The activity was determined from the oxygen consumption observed in a Warburg apparatus at 37.5°C in an oxygen atmosphere. The system contained 0.6 ml of 0.05 M lithium urate, 1.0 ml of 0.1 M borate buffer pH 9.0, 0.5 ml of the enzyme preparation, 0.2 ml of 2.5 M sodium hydroxide in the center well and sufficient water to make the total volume of fluid in the vessel 3.0 ml. In all cases activity was calculated as microliters of oxygen per milligram of liver

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TABLE I. Comparison of Liver Uricase Activity with Uric Acid and Allantoin Excretion during 7th Week.

Group	I	II	III
Total uricase activity*	10,766	36,708	53,566
Range	7,738–12,789	28,416–52,924	45,240–63,826
Compared with Group III			
% decrease	80	31	—
t value‡	8.4	1.0	
Compared with Group II			
% decrease	71	—	—
t value‡	4.2		
Allantoin†	696	1,217	1,617
Range	542–875	771–1,500	1,313–1,708
Compared with Group III			
% decrease	57	25	—
t value‡	18.8	8.3	
Compared with Group II			
% decrease	43	—	—
t value‡	6.4		
Uric acid†	5.8	10.1	12.0
Range	5.0–6.7	6.7–19.2	8.7–19.2
Compared with Group III			
% decrease	52	16	—
t value‡	7.1	2.7	
Compared with Group II			
% decrease	43	—	—
t value‡	1.7		
Ratio, uric acid/allantoin	.0086	.0087	.0074
Range	.0076–.0093	.0052–.0150	.0051–.0119

* μl of oxygen per day per liver per hr.

† mg per animal per day.

‡ Values greater than 2.6 are significantly different at the 5% level of probability. Values greater than 4.0 are significantly different at the 1% level.

nitrogen per hour, the nitrogen having been determined by Kjeldahl analysis. *Allantoin* was determined by the method of Young and Conway(4), modified for use with the photoelectric colorimeter. In these determinations the samples were read on the A scale of a Fisher colorimeter, using Filter 425 and water as a blank. By using a standard curve the readings were converted to milligrams of allantoin. *Uric acid* was determined colorimetrically by a modification of the method of Brown(5). The samples were read on a Fisher colorimeter, using Filter 650 and the A scale. All determinations were made in duplicate and the uric acid content determined from a standard curve.

Results. In all groups the allantoin excretion varied considerably from period to period, the high values for each group being 2 to 3 times that of the low values. The animals on the protein-free diet consistently excreted less allantoin than did the animals in the other 2 groups. Except for the first week, the excretion of uric acid was also lower for the animals on the protein-free diet.

The *ad libitum*-fed group which may be regarded as the control showed a variation in the uric acid-allantoin ratio from 0.006 to 0.016. This range included nearly all the values of the other 2 groups. During the sixth week the high value was exceeded by the protein-free group. However, the high ratio was not maintained and no significance can be attached to this exception. The wide variation of the ratio from week to week and the uniform variation among the 3 groups would suggest that this ratio is not a valid index of uricase activity.

Since the ratio of uric acid to allantoin was approximately the same for the 3 groups due to the fact that the uric acid as well as the allantoin content of the urine decreased, the possibility that the uricase activity could be predicted from either the uric acid or the allantoin content of the urine was considered. Table I shows a comparison of the total uricase activity of the liver, and the allantoin and the uric acid content of the urine for the seventh week for each group, as well as the relationship of the groups with respect to each of these factors. It is apparent that the decrease in allantoin content of the urine parallels the decrease in uricase activity. Thus the allantoin content of the urine may indicate the uricase level of the rat liver. However, the uric acid in the urine also parallels the decrease in uricase activity. Furthermore, the allantoin content of the urine varied considerably from week to week throughout the experiment. Further studies of these correlations would be required before it would be justified to regard the allantoin content of the urine as an index of uricase activity.

The results of these experiments suggest the possibility that the *in vivo* mechanism of the uricase reaction is comparable to the *in vitro* mechanism proposed by Kemperer(6) and Praetorius(7) who suggest that, on oxidation of uric acid by uricase, a primary reaction product is formed which in a subsequent non-enzymatic reaction gives rise to the final decomposition products: allantoin, hydroxyacetylene-diuride carboxylic acid and uroxanic acid. The relative amounts of these decomposition products depend on the pH of the medium and the nature of the buffer. If this comparison is valid then neither the allantoin nor the uric acid content of the urine would give an index of uricase activity.

Summary. Allantoin and uric acid have been determined in the urines of rats fed a protein-free and 18% casein diet. The animals on the casein diet consisted of a group pair-fed with the animals on the protein-free diet and a group fed *ad libitum.* The low uricase activity in the livers of the rats on the protein-free diet was not accompanied by an increase in the uric acid-allantoin ratio in the urine. Thus in the rat it is not possible to predict the *in vivo* uricase level from the uric acid-allantoin ratio in the urine. However, there is a correlation between the allantoin or the uric acid content of the urine and the uricase content of the liver. Whether there is a relationship between these factors can not be answered at this time.

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Effect of Cortisone upon Growth *in vitro* of Femur of the Chick Embryo.[*] (21896)

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In adequate doses, cortisone produces a distinct and rapid inhibition of growth in the rat(1), with marked abnormalities in the newly formed cartilage and bone(2). Suspension of treatment allows of a rapid recovery in weight(3). In other animal species (mice, guinea-pigs, rabbits) the inhibitory action of cortisone was also evident(4). ACTH also shows a marked inhibitory action upon growth of rats(5,6). An inhibitory effect upon growth has also been shown in the chick embryo(7,8).

It has not been shown whether such effects are the result of a direct action of the hormone upon bone growth or of an action indirectly through some other gland and/or the general metabolism, especially in view of the catabolic effect on proteins possessed by this corticoid.

Cortisone acts directly upon cells in cultures(9-13), on the segmentation of the egg of Arbacia(14); and on mastocytes *in vivo* (15) and *in vitro*(16).

In our studies, cortisone acted directly on bone *in vitro*, thus precluding all possibility of intermediate factors. This same technic has been employed to study the effect on bone growth of thyroxin, vit. A, growth promoting hormone and estradiol(17-20).

Material and technic. Femurs were used of Rhode Island chick embryos in their 7th day of incubation, and cultivated according to the technic of Fell, upon a clot in a moist chamber(18). The embryo was dissected and both femurs removed; one femur was cultivated in the medium containing hormone, the other femur being cultivated as a control in a medium without hormone. Hormone was added to the medium, producing a very fine suspension in the Tyrode at a concentration of 0.1 mg per ml. Cortisone acetate and hydrocortisone acetate were used in the final medium in a concentration of 0.025 mg per ml. Every 2 or 3 days the femurs were removed to a fresh clot, identical in composition to that used in the first explantation, and were measured with a micrometric eyepiece, the lengths being expressed in arbitrary units. In 2 series of experiments, the first and second explantations were made in a medium containing cortisone, but subsequently the successive transplantations were made without this

TABLE I. Effect of Cortisone on Increase in Length of Chick Embryo Femur, *In Vitro*.

Days after explantation	Series	Control No. of cultures	Control M ± E[*]	+ Cortisone No. of cultures	+ Cortisone M ± E[*]	Difference	P
2	a	11	3.86 ± .25	11	2.41 ± .28	1.45	.001
	b	14	4.93 ± .33	14	2.92 ± .24	2.01	.001
4	a	11	6.07 ± .29	11	4.16 ± .25	1.91	.001
	b	13	6.96 ± .36	13	4.21 ± .24	2.75	.001
6	b	13	9.03 ± .37	13	5.94 ± .31	3.09	.001
7	a	11	8.77 ± .35	11	5.61 ± .27	3.16	.001
8	b	7	10.03 ± .14	7	6.78 ± .43	3.25	.001
9	a	11	9.50 ± .34	11	6.43 ± .27	3.07	.001
10	b	4	10.12 ± .38	4	7.06 ± .95	3.06	.001
12	a	10	10.17 ± .4	10	6.92 ± .26	3.25	.001
15	a	7	11.50 ± .45	7	7.89 ± .33	3.61	.001
17	a	7	11.67 ± .52	7	8.03 ± .25	3.64	.001
19	a	3	11.66 ± 1.20	3	8.16 ± .33	3.50	.001

[*] M = arithmetical average. E = standard error.

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[*] We are indebted to Merck and Co., Rahway, N. J., and to their representative in Montevideo, Mr. A. Puig Sanchez, for the hormones used in this research.

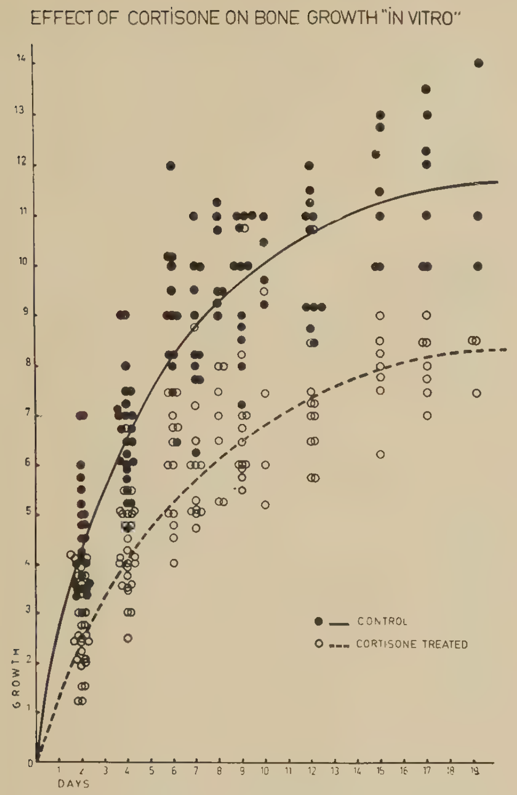

FIG. 1. Graph showing effect of cortisone on growth of chick embryo femur *in vitro*. Lines indicate average growth of control (solid line) and cortisone treated (dotted line). No matter which initial length of the bone was at the beginning of the experiment it has been taken as zero, and therefore the lines show daily increase of length.

hormone. All experiments were rejected in which deformities or curvatures appeared which could affect the results. In all, 59 experiments were conducted. At varying times after the first explantation, the material was fixed in formalin-calcium solution (Baker) and histological study made. Thus, in one series of experiments (a) the skeletal anlages were kept in a hormone-containing medium throughout, but with a varying duration for each individual experiment. In another series (b) the skeletal anlages were cultivated in a hormone-containing medium for only 48 hours, and then the culture was continued without hormone. The longest experimental series lasted 19 days, entailing 7 transplantations, the first 3 at 2-day intervals, the rest every 3 days.

Results. The results obtained in a total of 28 experiments, in which the anlage was kept in a medium with hormone throughout, are shown in the table and in Figs. 1 and 2. The data show that all the anlages of bones cultivated in a medium containing cortisone or hydrocortisone were inhibited in their growth. This effect was already evident at the moment of the first transplantation, *i.e.*, 48 hours after cultivation was begun. At this time, control bones had grown an average of 3.86 ± 0.25, while those treated had grown 2.41 ± 0.28. This difference increases until the 15th day after the beginning of the experiment, after which the difference reached does not vary materially. All the experiments carried out showed the same type of abnormality, although to a varying degree. This

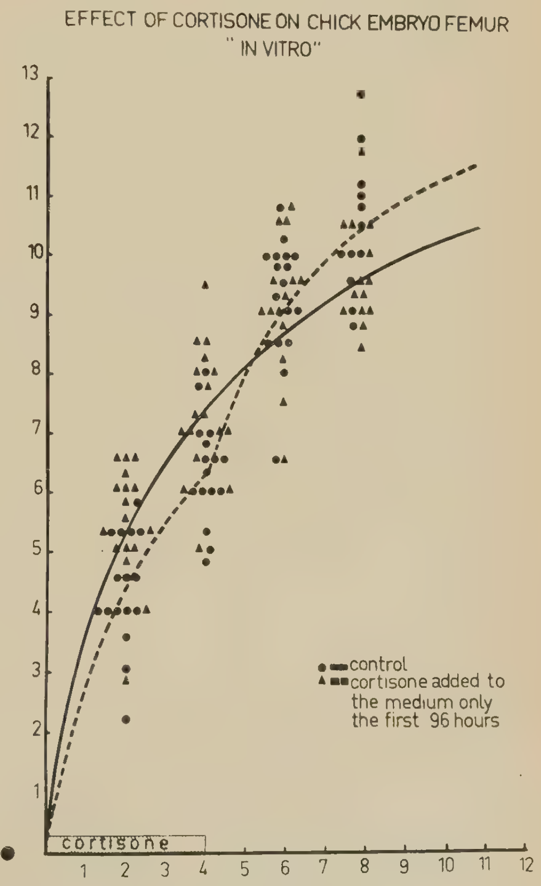

FIG. 2. Same as Fig. 1 but in this case cortisone was added to medium only in 1st and 2nd transplantation. After the 2nd transplantation (96 hr) both, control and treated, were transplanted to normal medium without cortisone.

fact, together with the actual values of the differences, makes the results highly significant. There was no appreciable difference between the effects of cortisone and hydrocortisone.

Quite an unexpected result was obtained in the experiments in which the cortisone or hydrocortisone was present in the explantation and was absent at the first or the second transplantation. Forty-eight or 96 hours after explantation, the anlages suffered the usual arrest in growth we have described above. On the other hand, when these treated femurs were transplanted to a fresh medium without hormone, growth began again at a very high rate, the final dimensions being, in some cases, greater than those of the controls (Fig. 2).

Histological study of the bone anlages carried out at variable times during cultivation, showed a normal, healthy appearance of the cells until the 10th or 12th day; after this, degenerative changes were seen both in the treated and the control femurs, their tissues being practically alike. Study of the lipids showed, within the cells, droplets made evident by the use of Sudan black, and which increased with the duration of the experiment. Employing the periodic acid-sulfofuchsine method, a weakly positive reaction appeared in the osteoid zone. Comparison of the histological appearance of the treated and the control anlages did not lead to any conclusions. Inhibition of growth does not appear to be due to a decrease in the size of the cells or in the amount of fundamental substance, but rather to a diminished proliferation of the former.

Discussion. The arrest of skeletal growth observed in experimentation *in vitro,* is in agreement with the results already mentioned, obtained *in vivo*. In the first case, it is evident that the cortisone acts directly upon the cartilage. We believe that the same conclusion can be applied to the action of the hormone in the intact animal, its action not being through the glands, such as the hypophysis, or through a general metabolic action. When the hormone is withdrawn, after a period of treatment, the rapid recuperation of growth is also similar *in vitro* and *in vivo*(3), although greater in the former case, wherein the dimensions of the treated anlage surpass those of the untreated control. The action appears to be a direct inhibition of cell proliferation, inasmuch as we have not seen cellular atrophy or reduction in the fundamental intercellular substance of the cartilage.

This marked action of cortisone upon the growth of the skeletal anlage, contrasts with the negative results obtained with the same technic, but using growth hormone(19); on the other hand, thyroxin and vitamin A are evidently active(17,18).

Summary. Cortisone and hydrocortisone act *in vitro* inhibiting the growth of explanted chick embryo femur anlages. This effect is evident 24 hours after explantation, and increases until the 15th day after the beginning of the experiment. When the hormone is withdrawn at the first transplantation, the anlage recovers active growth and in some instances surpasses the dimensions of the control.

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Comparative Effects of Muscle Work and Insulin upon Plasma Amino Acids in Eviscerated Rats.* (21897)

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Insulin will suppress the rise in plasma amino acids and enhance glucose tolerance in the eviscerate animal. Stimulation of muscle has an insulin-like effect upon the glucose tolerance of normal, diabetic and eviscerate animals(1). Under the conditions of the present experiments muscle work failed to simulate insulin in suppressing the rise in plasma amino acids following evisceration of the rat.

Methods. Male rats of the Sprague-Dawley strain were fed Archer Dog Pellets. At a weight of 250 ± 2 g non-fasted rats were anesthetized with cyclopal sodium and functionally eviscerated. The stimulation of muscle to contract 5 times per second was by the method of Ingle(2). The left hind leg was weighted with 100 g. The stimulus passed from the right hind foot to the left hind foot, thereby activating all of the musculature of both hind legs. A solution of glucose, with and without insulin, was infused into the jugular vein by a continuous injection apparatus which delivered fluid from each of 6 syringes at the rate of 20 cc in 24 hours. The experiment lasted 3 hours, whereupon the blood was collected by cannula from the abdominal aorta. Blood glucose was determined by the method of Miller and Van Slyke(3) and plasma amino acids by the method of Hamilton and Van Slyke(4).

Experiments and results. The conditions of each experiment are given in Table I. The data of Groups 1, 2 and 3 illustrate the characteristic rise in plasma amino acids following evisceration and its suppression by insulin. In Group 4, the stimulation of muscle greatly increased glucose tolerance but failed to suppress the rise in plasma amino acids. When insulin was administered with glucose there was partial suppression of the rise in plasma amino acids. The average value for plasma amino acids was lower in the insulin-treated resting rats than in the insulin-treated working rats; the difference between the means (2.20 ± 0.533) meets the usual requirements for statistical significance. The combination of insulin and muscle work (Group 5) had a greater effect upon glucose tolerance than either one alone.

Discussion. The relationship between the effects of insulin upon the utilization of carbohydrate and upon the metabolism of amino acids and proteins is not understood although it is commonly assumed that the effects of insulin upon protein metabolism are secondary to some primary action upon the utilization of carbohydrate. Both insulin and muscle work increase the transfer of glucose and other sugars into cells(5). It has been suggested (6) that the increase in the rate at which glucose enters the cell represents the primary action of insulin to which all other consequences of its action are secondary. Since muscle work simulates the action of insulin upon glucose utilization and glucose space, its failure to have an insulin-like action upon the rise of plasma amino acids following evisceration of the rat requires further explanation.

In confirmation of earlier studies(1) the data of Group 5 (Table I) show that a com-

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TABLE I. Comparison of Effects of Muscle Work and of Insulin upon Levels of Blood Glucose and of Plasma Amino Acids in Functionally Eviscerated Rats. Averages and standard errors.

						Work both legs			No work	
Group	Experimental condition	Time, hr	Load glucose, mg/100 g rat/hr	Insulin, units/24 hr/rat	No. rats	Total work	mg % PAA*	mg % BS†	mg % PAA	mg % BS
1	Normal	0	0	0	14				5.94 ± .17	130.9 ± 3.57
2	Eviscerated	3	0	0	13				10.61 ± .257	68.2 ± 4.78
3	”	3	0	16	13				4.49 ± .264	26.7 ± 1.97
4	”	3	110	0	12	8738 ± 611	11.24 ± .353	307.3 ± 31.26	11.31 ± .342	692.0 ± 37.3
5	”	3	110	16	12	7146 ± 447	6.87 ± .438	95.0 ± 10.1	4.67 ± .303	286.0 ± 26.4

* Plasma amino acids. † Blood sugar.

bination of optimal doses of insulin and maximal work has a greater effect upon glucose tolerance than either one alone. The failure to achieve optimal glucose utilization by work alone or by insulin alone is further reason to suggest that the effects of muscle work and of insulin upon metabolism are not identical in all respects. Moreover, in the present as in an earlier experiment(7), the administration of insulin to the working rat causes a suppression of work output (comparison of Groups 4 and 5) in those animals which are made hypoglycemic by the addition of insulin. It has been suggested(7) that insulin favors pathways of glucose utilization which compete with contracting muscle for the available carbohydrate.

Summary. Stimulation of muscle has an insulin-like effect upon the glucose tolerance of the eviscerate rat. In the rat the effect upon glucose tolerance of a combination of muscle work and of optimal doses of insulin is greater than of either one alone. Insulin suppresses the rise in plasma amino acids following evisceration of the rat but muscle work does not.

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Serum Lactic Dehydrogenase Activity as Indication of Neoplastic Growth and Regression.* (21898)

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Lactic dehydrogenase (LDH) activity has been studied in the tissues from normal and tumor bearing mice(1,2) in transplanted tumors(3) and in induced tumors(4). Although significant amounts of lactic, malic and isocitric dehydrogenases were found to be present in transplanted tumors(3,5), attempts to correlate LDH activity with tumor growth failed to reveal any difference in enzyme activity between organs of mice with and without certain transplanted tumors(2). In a tumor induced by painting 0.6% methylcholanthrene a difference was detected in LDH activity between neoplastic tissue and normal skin(4).

In human blood serum LDH has also been shown to have a consistent relationship to neoplastic disease(6). Except in the case of pregnancy, in which an elevation of LDH was reported(6), no difference in activity from normal has been observed in diabetes, cardiovascular diseases, tuberculosis, various miscellaneous diseases and in benign tumors.

In this report we present data confirming the observation of a direct relationship between LDH and tumor growth in mice, and based on this relationship, success in determining the onset and duration of the effect of transplanted tumors on the hosts.

Materials and methods. Male Swiss mice 1½ to 3 months old and male and female Leaden mice of the same age were kept in a large room maintained at about 80°F. Two kinds of tumors were used for transplantation —tumor D, a highly differentiated spontaneous squamous cell carcinoma from the leg of a Leaden mouse, was transplanted subcutaneously to both male and female Leaden mice and tumor #3, a comparatively undifferentiated squamous cell carcinoma induced by methylcholanthrene, was transplanted in the same way to male Swiss mice. The Swiss mice were inbred in our laboratory for 7 years. The numbers are given in brackets. 0.1 - 0.2 ml of blood was taken by heart puncture during the 30-day period after tumor transplantation. Serum was then separated from blood cells by centrifugation at 4°C. Recentrifuging the serum removed any trace amounts of blood cells which might be present in the serum. Samples showing hemolysis were discarded. Serum was diluted 50 times with redistilled water just before determination of LDH activity. One lambda of serum was sufficient for each determination by the micromethod of Strominger and Lowry(7).

TABLE I. LDH of Sera of Leaden Mice with Tumor D Transplants.

Days after tumor transplantation	LDH (mM substrate split per ml serum per hr) Male Range	Male Mean	Female Range	Female Mean	% increase over control M	F
Control	25.4– 41.4 (7)	34.0	25.6– 33.0 (3)	28.8		
1	26.4– 77.2 (6)	40.0	30.0– 33.0 (2)	31.6	17.7	7.7
2	19.2–109.4 (3)	77.4	90.2–121.8 (3)	103.4	127.9	259.0
3	118.2–122.0 (2)	120.2	119.0–135.6 (3)	125.8	253.5	336.8
6	122.8–159.2 (3)	136.8	—	—	302.3	—
9	90.0–151.8 (5)	119.2	114.0–157.8 (6)	136.6	250.7	374.3
12	124.4–138.4 (3)	131.0	—	—	285.3	—
15	107.4–174.6 (6)	144.0	149.8–167.6 (2)	158.8	323.5	465.3
18	138.2–255.2 (5)	207.0	154.0–205.8 (4)	177.2	509.0	515.3
21	126.0–198.2 (5)	163.6	157.8–269.0 (6)	224.4	381.2	679.2
24	183.2–211.8 (4)	195.4	133.0–204.4 (3)	174.8	474.7	506.9
30	246.8–370.8 (7)	291.4	223.8–377.0 (8)	285.8	757.1	892.4

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* Aided by the Charles F. Kettering Foundation and an Institutional Grant from the American Cancer Society.

TABLE II. LDH of Sera of Swiss Male with Tumor #3 Transplants.

Days after tumor transplantation	LDH (mM substrate split/ml serum/hr)		% increase over control
	Range	Mean	
Control	92.6–145.6 (4)	124.2	
9	165.6–202.8 (3)	184.6	48.5
15	265.6–307.6 (3)	280.8	126.1
21	197.4–346.4 (3)	292.2	135.3
30	383.6–387.2 (2)	385.4	210.3

Results. Average values of serum LDH of Leaden mice transplanted with tumor D are shown in Table I. Two days after tumor D was transplanted an elevated serum LDH was seen in both male and female Leaden mice. The rise in serum LDH was continued during the whole period of experimentation. Several interesting modifications occurred with the lapse of time. First, there was the sudden increase of LDH approximately from 1-3 days after transplantation. A relative plateau covered the second period from 6-15 days; and then the third period, from 18-30 days, was manifested by a resumed sharp rise of serum LDH. It is noticeable that a slight drop in LDH activity was located in the last stage roughly around 20 days after tumor transplantation for both sexes. This dip in activity was a temporary one, only to be superseded by a higher level afterwards.

Table II shows the increase of serum LDH of mice transplanted with another kind of epithelial tumor. Although fewer determinations were carried out in this experiment, the general trend was the same, namely, the constant rise in LDH activity during the whole period, characterized by sharp increases in the first and third stages as described previously, and a possible drop around the 20th day. It should be pointed out here that the over-all level of LDH activity was considerably less than that given by the Leaden mouse serum with transplanted tumor D, being roughly one-fourth of the latter.

Table III shows serum LDH with regressed tumors D and #3. Brief mention should be made here of the general properties of these 2 tumors. Tumor #3 is a slower-growing transplant than tumor D. About 10-15% of regression have been observed in transplantation after 77 generations. The regression in tumor D in female mice was the first such case observed in a total of 106 generations. It is readily seen that in both instances regression brought back the LDH concentration to lower levels, and, furthermore, in the case of the female Leaden mice implanted with tumor D, resumption of tumor growth after temporary regression again increases LDH activity.

Discussion. Considering the results as a whole, some broad lines may be drawn in describing the relationship between tumor growth and serum LDH activity in mice. 1. Serum LDH activity generally follows the tumor growth; in other words, LDH activity increases as the tumor ages. The absolute amount of LDH may vary with species, kind of tumors, age, and perhaps other factors, but the relationship holds true at least in the case of the tumors and the mice we have investigated. 2. Transplantation of tumors brings about an immediate change in serum LDH (and possibly other metabolic changes) which

TABLE III. LDH Sera of Mice Having Regressed Tumors.

Condition	LDH (mM substrate split per ml per hr)		
	Range	Mean	
Control	92.6–155.6 (4)	124.2	
Male Swiss mice with regressed tumor #3 (1 week after regression)	114.8–143.4 (3)	132.2	21 days after transplantation, tumor regressed
Control	25.6– 33.0 (3)	28.8	
Female Leaden mouse with regressed tumor D (3 days after regression)	128.2 (1)	128.2	15 days after transplantation, tumor regressed
(15 days after regression)	117.2 (1)	117.2	
(25 days after regression, tumor regrew)	212.2 (1)	212.2	

is readily demonstrable within 2-3 days after transplantation. The magnitude of this alteration is so great as to make its detection unmistakable. 3. The serum LDH activity approximates to normality when tumor regression is near completion so that its reliability as an indicator is evident.

Table III indicates that the process of regression can be followed by determinations of serum LDH activity. The length of time within which LDH returns to normal seems to be dependent on differences in the properties of the tumors transplanted. Sera of animals carrying tumor D apparently take a much longer time than those carrying tumor #3 to return to the normal enzyme level. It is possible that residual tumor cells persist in these animals and are responsible for the regression.

The slight drop in LDH during the time period may be a naturally biological phenomenon. Cyclic changes in growth rate are not unusual and if present here could underly the alterations in LDH. Sex difference apparently influences this decline, as can be seen from Table I. In females it occurred at the 24th day, and in males at the 21st day. In the case of Swiss male mice transplanted with tumor #3, it apparently occurred also at the 21st day. The fact that it preceded an even sharper rise in LDH may be clarified by further studies.

Thus the response of serum LDH to the implantation is seemingly dependent on the kind of tumor implanted and on the sex of the mice. The promptness of this response provides an opportunity to make chemical investigations of small amounts of tissue before they show morphological evidence of growth.

Summary. Lactic dehydrogenase activity of the sera of Swiss and Leaden mice increases promptly after tumor transplantation and decreases with tumor regression.

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Disturbance of Pregnancy in Mouse and Rat by Systemic Antihistaminic Treatment. (21899)

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Histamine antagonists, which were instilled into the lumen of the uterus, inhibited the development of deciduomata in the traumatized endometrium of the pseudopregnant rat(1,2). These findings suggested the need to explore the action of antihistamines on the course of natural pregnancy, especially on implantation of the blastocyst. We report experiments demonstrating that antihistaminic drugs injected subcutaneously into the mouse and into the rat interfere with the natural course of gestation.

Methods. The antihistamine, Benadryl, was tested on 95 mice, and the histamine antagonist Pyrrolazote on 44 rats. All animals were housed in air-conditioned quarters, fed Purina laboratory chow with weekly supplements of seasonal greens and with fresh water *ad lib.*

Studies on mice. Series I was designed so that antihistamine injections were given during estrous cycle, or after mating, fertilization, implantation, and early pregnancy. *Methods.* Swiss albino mice (19-21 g) were used; 90 females, 10/cage were placed with males previously tested as fertile. Of the 10, 5 were given daily subcutaneous injections of 1 mg antihistamine Benadryl (diphenhydramine) in normal saline; the other 5, given injections of drug-free saline, were controls. After 10 injections, the males were removed. *Results.* In the antihistamine treated group 6 of 45 became pregnant and had normal litters, while in the saline control group there were 24 of 45 normal pregnancies. Series II was designed to test the action of Benadryl on early pregnancy, after normal mating. *Methods.* In this series, 100 mice of the same colony as Series I, were placed 4/cage with one fertile male. The male was removed after 5 days. Benadryl injections of 1 mg were given for 6 days to 2 of 4 mice per cage. The other 2 mice were controls which received only saline. Treatment was stopped and observation carried on for 25 days. *Results.* Of 50 antihistamine treated mice 3 became pregnant, compared with 10 of 50 controls. Since animals in this series were with males only half the number of days that those of Series I, the opportunity for coition was reduced one-half. Thus to compare results of series I and II, in respect to sexual contact opportunity, series II should be adjusted to read: experimental, 6 of 50 pregnant; control, 20 of 50 pregnant. From the combined series I and II only 13% of the antihistamine treated mice had normal pregnancies, compared to 50% of the controls. Beyond the fact that the drug acted after mating, the mechanism of Benadryl interference with normal pregnancy in the mouse remained undefined.

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* At the Israeli Institute for Biological Research, Ness-Ziona, during the performance of these experiments.

Studies on rats. The Pyrrolazote was selected for injections into rats, because we previously found it the most effective of 7 antihistamines tested in respect to inhibition of decidual cell reaction in the rat(2). Series I. This series was designed to find out at which stage of gestation the antihistamine was active in respect to disturbing pregnancy. *Methods.* Of 50 colony bred albino rats (160-210 g), 25 were Pyrrolazote treated, and 25 were controls. All were mated at proestrous, and were considered impregnated if the vagina contained sperm. The animals were grouped into 5 sets which differed in respect to the period of gestation during which they received, twice daily, subcutaneous injections of 15 mg Pyrrolazote, or of saline. Set 1 was injected on days 4, 5, and 6 after coition; set 2, on days 5, 6, and 7; set 3, on days 6, 7, and 8; set 4, on days 7, 8 and 9; and set 5 on days 8, 9, and 10. Observations were continued through gestation. *Results.* From Table I evidence indicates that the Pyrrolazote interferes with normal development of pregnancy; and the drug action is greatest when given after implantation. *Series II* was designed to examine the condition of pregnant uteri after Pyrrolazote injections. *Methods.* In this series 27 female rats (160-200 g) were mated at proestrous; impregnation verified by the presence of sperm in the vagina. This group included 19 test animals and 8 controls. Test animals were injected twice daily with 15 mg Pyrrolazote on days 6, 7, and 8, after coition. Beginning on the 8th day of pregnancy and continuing through day 15, uteri of experimental and control animals were removed. *Results.* The uteri of Pyrrolazote showed no normal pregnancies (Fig. 1); and microscopic examination of these uteri revealed various stages of fetal absorption, and abortion; in one case massive decidual reaction without evidence of any embryos. Except for the case of the deciduoma, all uteri showed that implantation had occurred, but there was subsequent interference with fetal development.

TABLE I. Action of Pyrrolazote on Gestation in Rat.

Treatment: Subcut. inj. 15 mg × 2/day × 3 days.

Days, post-coition, injected	4,5,6	5,6,7	6,7,8	7,8,9	8,9,10
Experimental: pregnant/total	4/5	2/5	1/5	2/5	0/5
Saline controls: pregnant/total*	5/5	5/5	5/5	5/5	5/5

* In this group of 25 females, all inseminated rats carried to normal term; however the conception rate of this colony (vaginal sperm/normal litter) is >97 $<98\%$, measured in over 600 cases.

Discussion. The effectiveness of injection of antihistaminic drugs into the uterus in suppressing deciduoma formation in the rat(1,3),

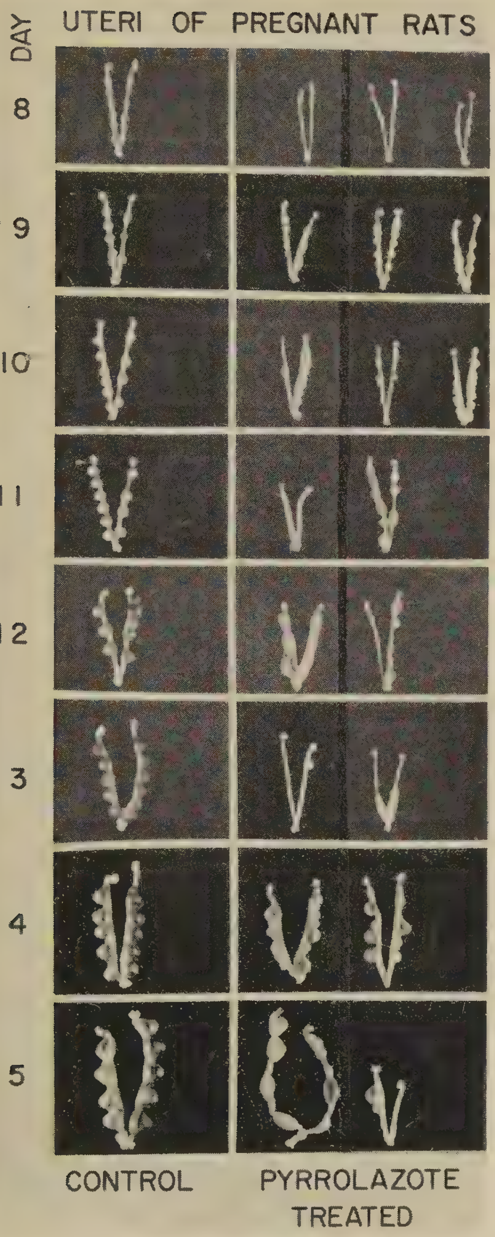

FIG. 1. Whole uteri of pregnant rats, at gestation days from 8 to 15. Left hand column shows saline controls: normal size and distribution of fetal sites. Right hand column shows abnormalities, deciduomal sites, fusions, abortions and resorptions.

suggested the study of such drugs on normal pregnancy. If one assumes that the drug action which suppressed the artificially induced decidual cell reaction, also suppressed the natural decidual reaction occurring at the time of blastocyst implantation, then treatment with antihistamine should prevent pregnancy by inhibiting normal implantation. The artificial decidual reaction was inhibited with definite quantities of antihistaminic drugs instilled directly into the uterus(2). This procedure could not be directly adopted to study pregnant animals, since the free-floating fertilized eggs could be damaged, and no evidence of drug action on decidual reaction would exist. Systemic route of drug administration was used.

The failure of systemic antihistamines to prevent *implantation* in these studies (and 4) may be considered due either to the failure of the drug to inhibit natural decidual growth, or to the failure of an adequate amount of the drug to reach the uterus following systemic injections. It becomes strikingly clear that an insufficient quantity of antihistamine reaches the uterus, if the drug is given via subcutaneous injection(5,6).

In the rat we showed that .2 mg Pyrrolazote was needed *in utero* to prevent deciduoma formation. Calculating the amount of pyrrolazote which reached the uterus after 15 mg subcutaneous injection, on the basis of Benadryl(5) and Pyribenzamine(6) injections in the rat, we find only 1/100th of the required dose; and the 15 mg dose is very large. In fact these experiments could not test the action of subcutaneous antihistamine on natural implantation decidua, since an effective quantity of drug, as judged from artificial deciduoma studies(2), did not reach the endometrium.

The quantity of antihistamine which reached the developing fetus was however adequate to produce damage, and did disturb fetal development. There was a significant decrease in normal pregnancies.

Summary. Subcutaneous injection of the antihistamine Benadryl into 95 female mice, and of Pyrrolazote into 44 female rats, significantly reduced the number of normal pregnancies. The antihistamine acted on the developing fetus, after implantation had occurred.

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Changes in Estrous Cycle of Rat During Prolonged Exposure to Cold.* (21900)

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Previous investigations on the effect of exposure to cold on the estrous cycle of the rat were concerned only with short periods of exposure(1,2) and gave contradictory results (3). Since it is possible to maintain a certain percentage of female rats at an ambient temperature of 2 ± 2°C for as long as 90 days(4), the present study was undertaken to examine the effect of prolonged exposure to cold on the estrous cycle of the young adult rat.

Materials and methods. Approximately 50 female rats of the Harvard-Wistar strain were used. The animals were kept at 23°C prior to exposure and received food and water *ad libitum*. During exposure to cold the animals were placed in individual wire cages without bedding and kept in a room maintained at a temperature of 2 ± 2°C. All animals were exposed to 12 hours of light daily, both prior to and during the experiment, since it has been reported that continuous light will affect the estrous cycle(5,6).

The duration of each stage of the estrous cycle was determined in 3 groups of rats by the vaginal smear technic and recorded as a percentage of total length of the cycle. Each group was studied for a period of approximately one month at room temperature (23°C) and then placed in the cold room for periods of up to 90 days. The first group of rats received no treatment while the other 2 groups were injected with 25 and 50 μg of thyroxine respectively. The hormone was dissolved at a slightly alkaline pH and injected daily intraperitoneally.

Results and discussion. The average length of each stage of the estrous cycle of the rat was determined both prior to and during the period of exposure to cold. Marked changes were observed in duration of various stages of the estrous cycle and a definite increase in total length of the cycle was noted during the cold exposure period. Duration of proestrus and estrus was increased whereas a marked decrease in length of diestrus and a less severe decrease in the metestrous period was noted. The average total length of the estrous cycle increased from 4.3 days at room temperature to 5.7 days during the period of exposure to the cold (Table I).

Administration of 25 μg of thyroxine to rats during the period of exposure to cold was partially successful in preventing appearance of an abnormal, estrous cycle. The average length of the cycle was not affected since cold-exposed rats treated with 25 μg of thyroxine showed a cycle of 5.9 days as compared to 5.7 days for cold-exposed rats not treated with thyroxine. Duration of the proestrous period

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TABLE I. Changes in Length of Estrous Cycle and Per Cent Duration of Each Stage.

Treatment	Length of estrous cycle, days	% of estrous cycle			
		Diestrus	Proestrus	Estrus	Metestrus
Room temperature	4.3	25	24	27	24
Cold	5.7	4	42	37	17
" —25 μg thyroxine	5.9	13	25	39	23
" —50 " "	4.7	20	25	29	26

was decreased as compared with the length of the period noted for the animals exposed to cold and was not significantly different from the value obtained for control rats kept at room temperature. In addition, treatment with this dosage of thyroxine had no effect on the estrous stage but caused a marked increase in length of diestrous phase and a slight increase in metestrous stage as compared with duration of these stages in rats exposed to cold alone.

Treatment of rats in the cold with 50 μg of thyroxine daily prevented the derangement of the estrous cycle noted on exposure to a low temperature. Total length of the cycle was 4.7 days in this group of animals, which is not significantly different from the 4.3 days found at room temperature. The proestrous phase of the cycle occupied 25% of the total period as compared to 24% at room temperature. The estrous phase occupied 29% of the total in cold as compared with 27% at room temperature. Similarly duration of both metestrous and diestrous stages was normal in all animals exposed to a temperature of 2 ± 2°C and treated daily with 50 μg thyroxine.

The present results, indicating a derangement of the estrous cycle of the rat following exposure to cold, confirm an earlier experiment by Dempsey and Uotila(2). The latter reported an increase in the length of the estrous cycle of rats exposed to a low temperature with a specific increase in duration of estrous and diestrous periods. They attributed this to an increased release of FSH or a delay in release of LH. A more recent investigation by Dempsey and Searles(3) indicated that increased length of the estrous cycle in cold was the result of constant light rather than due to a change in environmental temperature. In the present experiment, the effect of light on the length and type of estrous cycle was eliminated since the animals were maintained under a daily regime of 12 hours of light and 12 hours of darkness. Our results indicate an increase in duration of the estrous period, comparable to the results obtained by Dempsey and Uotila(2) but a decrease in the diestrous period which is contrary to their results. It may be concluded from our data that exposure to cold resulted in an increase in length of proestrus and estrus and a reduction in the length of the diestrous phase. The overall effect was a marked increase in the total length of the estrous cycle.

Several investigators have reported a relationship between the activity of the thyroid gland and the gonads(7-10). Many of these reports are controversial although some of them indicate an increased production of FSH under conditions that would induce an increased output of TSH. In view of the above and the fact that increased amounts of thyroxine are undoubtedly needed during exposure to a low ambient temperature, thyroxine was administered to female rats in an attempt to maintain a normal estrous cycle.

Treatment with 50 μg of thyroxine daily prevented the abnormal changes seen in the estrous cycle following exposure to cold. The mechanism whereby the thyroxine prevented the abnormal changes can not be determined from these experiments. It is possible that thyroxine was effective simply by virtue of its ability to increase the metabolic rate and thereby combat the effects of cold exposure; or it is possible that the increased exogenous thyroxine helped to maintain normal pituitary activity by preventing the increased TSH release due to the cold exposure.

Summary. Studies on the reproductive activity of the adult female rat indicate a marked increase in length of the estrous cycle following exposure to a low ambient tempera-

ture. Duration of both the proestrous and estrous stages was increased whereas length of the metestrous and diestrous stages was decreased. Treatment with 50 μg of thyroxine prevented the abnormal changes in the estrous cycle during exposure to cold.

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Effect of ACTH on Protein Synthesis in Isolated Perfused Beef Adrenal Gland.*† (21901)

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The observation that mammalian adrenal glands undergo marked hypertrophy while under the influence of adrenocorticotrophic hormone has been well substantiated. Smith (1) first produced such an effect by implanting pituitary tissue into hypophysectomized rats. Since this classical experiment, many investigators have studied the nature of this pituitary-adrenal relationship. In spite of extensive studies of the direct effect of ACTH on the adrenal gland *per se,* there has been no published report evaluating the qualitative nature of this tissue growth. This study was undertaken to determine whether ACTH stimulation may be related to protein synthesis as estimated by the incorporation of C^{14} labeled lysine into the adrenal gland proteins.

Methods. Isolated beef adrenal glands were perfused in parallel in an oxygenated atmosphere at 38-40°C with homologous blood containing ϵ-C^{14}-DL-lysine as well as essential and non-essential amino acids (Table I). The perfusion apparatus and procedure used, and the methods of working up tissue and plasma proteins to remove non-protein C^{14} contaminants were the same as those previously described(2). The arrangement of the apparatus was modified in that the lung and the overflow reservoir were elevated to a position yielding a perfusion (arterial) pressure of 120-130 cm of blood. In addition no carbon dioxide trap was used to collect "expired" gases.

Collection and preparation of blood. While the glands were procured, approximately one liter of blood was collected, non-aseptically from the severed neck vessels of an animal, in a 5-liter Erlenmeyer flask containing glass beads. The blood was then defibrinated by shaking vigorously for 15 minutes. At the laboratory 300-400 ml of this blood was strained through surgical cotton gauze sponges to remove clots. The blood was then transferred to the reservoir of the perfusion apparatus and circulated. To this blood was added the radioactive lysine and the supplements listed in Table I.

Collection and preparation of glands for

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TABLE I. Experimental Conditions.*

	No. of glands perfused			Amino acids added				
				Unlabeled†		Labeled ‡		
Exp. No.	Control period, G-1	Exp. period, G-2;3	Amt of perfusate, blood in ml	Essential, mg	Non-essential, mg	volts/min.	ACTH,§ mg	Duration of exp., hr
AP- 1	1	2	300	160	167	383	5 A	4
AP- 2	1	1	300	160	167	383	5 A	4
AP- 3	1	2	300	240	167	383	5 A	4
AP- 4	1	2	300	240	167	383	5 W	4
AP- 5	1	2	300	240	167	383	40 W	4
AP- 6	2	2;2	400	160	167	294	100 W + 5 A	6
AP- 7	2	3	400	0	0	294	15 A	4
AP- 8	1	2	300	160	167	383	0	4
AP- 9	2	2	400	0	0	294	0	4
AP-10	0	0	300	240	167	383	0	4

* In all experiments, 500 mg of glucose in physiological saline were added to perfusate before perfusion was begun; and 100% O_2 was used for oxygenation of blood.

† Proportions, preparations, and sources are found in reference 15.

‡ Labeled amino acid was administered as ϵ-C^{14}-DL-lysine. 1 volt per min. $= 1.88 \times 10^4$ disintegrations per min. Twice as many volts of DL-lysine were actually given, but it was assumed that only the L-isomer of the active lysine is utilized for protein synthesis. Calculations were based upon this latter assumption.

§ A denotes Armour's Porcine ACTH (lyophilized extract). W denotes Wilson's (ACTH) corticotropin solution in 0.5% phenol.

perfusion. Adrenal glands were removed from hanging carcasses of exsanguinated, skinned, and eviscerated cattle approximately 15-20 minutes after slaughter. During the collection period, the glands were kept in a beaker surrounded by an ice-salt mixture. After 10 minutes for transportation to the laboratory, the glands were trimmed of surrounding fat and connective tissue and the adrenal vein cannulated after ligation of any accessory veins present. After cannulation, the cortex was multiply scored to a depth of approximately 1-2 mm with a scalpel; then heparinized physiological saline was introduced into the cannula to prevent clots and expel air bubbles before the glands were attached to the perfusion manifold.

Perfusion of the glands. The glands, prepared as described above, were connected to a common manifold and perfused. All but one of the experiments were of 4 hours duration. Typically, the glands were perfused for a control period of 2 hours (Table II), during which no ACTH was added, followed by an experimental period of 2 hours, at the onset of which ACTH was added to the perfusate. The proteins of control glands removed after the first 2-hour period were assayed for C^{14} activity due to protein synthesis (measured as ϵ-C^{14}-L-lysine uptake) and compared to the activity of the gland protein after the experimental period. Hourly blood samples of 10-15 ml were also removed in order to allow a comparative examination of the C^{14} activity of the plasma proteins before and after ACTH addition. Blood samples of 100 ml were removed at the end of both the control and experimental periods and assayed for a comparison of corticosteroid hormone output.

Two experiments, No. AP-8 and AP-9 were carried out in which the glands were perfused for the total 4-hour period without ACTH. Gland, blood, and plasma samples were analyzed as above.

In order to determine if plasma protein became labeled with C^{14} in the absence of gland tissue, the blood was simply circulated through the apparatus under the usual conditions for the total perfusion period except that no glands were used in AP-10 (Table I). The proteins of serial plasma samples were assayed for C^{14} activity.

In the experiments with ACTH, 2 currently available commercial preparations (both Armour's and Wilson's preparations) were used and their effects compared.

Preparation of gland proteins and analysis for C^{14} activity. The glands, having been

TABLE II.* Adrenal Gland Protein Synthesis.

Exp. No.	Gland sample No.	1 Perfusion time in hr and amt of ACTH† added in mg	2 Total amt of dry protein in glands, g	3 ϵ-C^{14}-lysine activity in glands, v/m/g‡	4 Δ activity,§ v/m/g	5 % dose of ϵ-C^{14}-lysine incorporated into protein,‖ per g	6 Periodic ratio Exp. (G-2)/Cont. (G-1)	7 Corticoid output¶
AP-1	G-1	2–	1.84	.57		.95		+ 1
	G-2	2–, 2 + 5 A	4.50	2.14	1.57	2.94	3.1	+ 5
AP-2	G-1	2–	2.05	2.89		2.79		+ 1
	G-2	2–, 2 + 5 A	1.64	17.68	14.79	10.64	3.8	+ 5
AP-3	G-1	2–	1.66	.35		.51		**
	G-2	2–, 2 + 5 A	3.91	2.19	1.84	3.20	6.3	
AP-4	G-1	2–	2.11	.40		.68		**
	G-2	2–, 2 + 5 W	4.43	.75	.35	.44	.6	
AP-5	G-1	2–	1.65	.14		.17		+ 1
	G-2	2–, 2 + 40 W	3.07	.83	.69	.97	5.7	+ 1
AP-6	G-1	2–	4.65	1.13		6.62		+ 1
	G-2	2–, 2 + 100 W	5.67	1.19	.06	.34	.05	+ 2
	G-3	2–, 2 + 100 W, 2 + 5 A	6.85	1.33	.14	.63	1.8	+ 6
AP-7	G-1	1–	3.38	.07		.21		+ 1
	G-2	1–, 3(1 + 5 A)	5.33	2.20	2.13	5.67	27.4	+10
AP-8	G-1	2–	1.81	3.75		7.22		+ 1
	G-2	4–	5.56	5.31	1.56	12.98	1.8	+ 1
AP-9	G-1	2–	3.98	2.76		7.47		**
	G-2	4–	3.80	9.55	6.79	12.88	1.7	

* See text for elaboration of table.

† 2– denotes 2 hr with no ACTH. 2 + 5 A denotes 2 hr with 5 mg Armour's ACTH. 2 + 40 W denotes 2 hr with 40 mg Wilson's ACTH.

‡ Volts per min. per g of dry adrenal gland protein.

§ Difference between experimental (G-2) and control periods (G-1) (Column 3).

‖ Calculated from amt of initial dose of ϵ-C^{14}-L-lysine incorporated per g of dry adrenal gland protein.

¶ See text and Table III.

** No hormone analysis.

weighed and then frozen after perfusion, were allowed to thaw at room temperature, after which they were homogenized with distilled water in a Waring Blendor for 10 minutes. An aliquot was then treated three times with trichloracetic acid, and carrier DL-lysine, and finally with acetone according to a previously published procedure(2). After drying at 50-60°C, the dry protein was ground to a fine powder, and a weighed aliquot assayed for C^{14} activity in the apparatus designed and described by Bale(3).

Plasma protein analysis. The hourly blood samples were centrifuged for 40 minutes at 2,000 rpm, the hematocrits recorded, and the plasma removed from the red blood cells. The plasma samples were then treated as described above except that the acetone treatment was omitted. Five ml portions of the plasma proteins were then assayed as above for C^{14} activity.

Extraction of corticosteroids. The procedure used was that developed by Axelrod and Zaffaroni(4). The method consists of an 8-day dialysis of the blood samples which had been diluted—1:1:1, distilled water:absolute methyl alcohol:blood—and dialyzed against 40% methyl alcohol.

The dialysate was diluted with an equal volume of water and extracted with a volume of CHCl$_3$ corresponding to 15% of the total volume of diluted dialysate. The extract was then reduced to dryness *in vacuo* under nitrogen, at 40°C.

Chromatography. The dialysate extracts were chromatographed using the technics and apparatus developed by Zaffaroni and coworkers(5-7). The samples were applied to

8-cm wide paper strips after impregnation of the latter with either formamide:methanol (1:1), or propylene glycol:methanol (1:1). The strips were then developed in benzene (formamide saturated) or toluene (propylene glycol saturated) for 3 hours or 48 hours respectively. As previously described, triphenyltetrazolium chloride (TPTZ), iodine in potassium iodide, and concentrated sulfuric acid were used as reagents for the detection of the corticosteroids(6).

Results. Table II shows the gland protein C^{14} activity before and after ACTH stimulation. Column 1 lists the specific perfusion time and amount of ACTH acting upon each gland sample. Thus AP-1, AP-2, and AP-3 are perfusions in which the control glands (G-1) were perfused for 2 hours with no ACTH while the experimental glands (G-2) were perfused, for 2 hours longer after the addition of 5 mg of Armour's ACTH. In the 3 experiments, the experimental glands incorporated respectively 3.1, 3.8, and 6.3 times as much ϵ-C^{14}-L-lysine per gram of dry gland protein as did the control glands. This increase in protein synthesis was directly correlated with a pronounced rise in hormone output during the experimental period in the first 2 experiments (See column 7).‡ No analysis for adrenal hormones was done in AP-3.

AP-7 is unique in that 15 mg of Armour's ACTH was used instead of only 5 mg as in the other perfusions. Hence G-1 was perfused for one hour with no ACTH, while G-2, in addition to this, was perfused for 3 additional hours with 5 mg of ACTH added at the start of each hour period. During the latter 3-hour period 27 times more ϵ-C^{14}-L-lysine was incorporated over the former one-hour period. This would suggest that the extra hourly ACTH had an added effect upon protein synthesis. Although there was a comparatively larger output of hormone during the total G-2 period, it was only slightly more than was produced during the experimental period of other perfusions. In view of this, the comparative effect on protein synthesis was striking.

The observed additive effect of the hourly addition of ACTH may be explained by the finding of Geschwind and Li(8) and Pincus, *et al.*(9) that adrenal tissue and mammalian blood are capable of inactivating ACTH. Thus it is likely that the effect observed on protein synthesis in the perfusions in which a single addition of ACTH was made was not the result of a sustained action but more likely a progressively decreasing effect because of ACTH inactivation with time.

In 2 experiments, AP-8 and AP-9, no ACTH was used and the G-2 activity was respectively only 1.8 and 1.7 times as great as the G-1 activity. Analysis of AP-8 blood also showed no difference in hormone output between the G-2 and G-1 periods demonstrating a lack of cortical stimulation, as well as the observed lack of increased protein synthesis, in the absence of ACTH. These 2 experiments indicate that C^{14} labeled lysine was incorporated into protein at a comparatively low basal rate. The fact that in the G-2 glands C^{14} incorporation was approximately twice the value noted for G-1 gland protein suggests that the actual rate of incorporation was constant during these perfusion experiments.

It is noteworthy that the results of the experiments utilizing Armour's ACTH were consistent in the observed effects on both protein synthesis, measured as C^{14} lysine uptake, and hormone output. This was not the case, however, when Wilson's ACTH was used. Thus the experimental conditions in AP-4 were identical to those in AP-1 except that 5 mg of Wilson's ACTH was used instead of Armour's ACTH, during the G-2 period. Analysis of the latter sample in AP-4 showed a decline in protein synthesis indicating a lack of ACTH effect. No analysis for adrenal hormones was done in AP-4.

AP-5 was a repetition of AP-4 except that 40 mg of the Wilson's ACTH was used instead of 5 mg. Here G-2 showed an increase in activity 5.7 times that seen in G-1, but this was not correlated with an increased hormone output. Thus in this experiment the 40 mg of Wilson's ACTH produced an activity in protein synthesis roughly equivalent to the activity observed after the addition of 5 mg of Armour's ACTH but no cortical stimulation

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‡ For an elaboration of this column, see Table III.

TABLE III.* Chromatography of Blood Sample Extracts.†

	1	2	3	4	5	6	7
Exp. No.	Blood sample No.	Time and conditions‡	Gland activity % dose 2-C^{14}-L-lysine incorporated per g dry protein	Ratio of gland activities§	Comparative corticoid activity (visual)	Hormone output ratio $\frac{\text{BS-2(or 3)}}{\text{BS-1}}$	Adreno-cortical hormone types resolved
AP-1	BS-1	2–	.95	3.1	1	5	$C_{21}O_5$
	BS-2	2–, 2 + 5 A	2.94		5		$C_{21}O_4$
							$C_{21}O_3$
AP-2	BS-1	2–	2.79	3.8	1	5	$C_{21}O_5$
	BS-2	2–, 2 + 5 A	10.64		5		$C_{21}O_4$
							$C_{21}O_3$
AP-5	BS-1	2–	.17	5.7	1	1	$C_{21}O_5$
	BS-2	2–, 2 + 40 W	.97		1		$C_{21}O_4$
							$C_{21}O_3$
AP-6	BS-1	2–	6.62	.05	1	2	
	BS-2	2–, 2 + 100 W	.34		2		$C_{21}O_5$
	BS-3	2–, 2 + 100 W, 2 + 5 A	.63	1.84	6	6	
AP-7	BS-1	1–	.21	27.35	1	10	$C_{21}O_5$
	BS-2	1–, 3(1 + 5 A)	5.67		10		$C_{21}O_4$
							$C_{21}O_3$
AP-8	BS-1	2–	7.22	1.80	1	1	$C_{21}O_5$
	BS-2	4–	12.98		1		$C_{21}O_4$
							$C_{21}O_3$

* Hormone output was not analyzed in AP-3, 4 and 9 since conditions in these experiments very closely paralleled those in AP-1, 2 and 8 respectively.

† Chromatographic resolution effected in toluene (propylene glycol saturated) and benzene (formamide saturated) systems.

‡ See Table II, †.

§ See Table II, column 6.

of hormone production beyond the control level was observed.

In AP-6 100 mg of Wilson's ACTH was added and the experiment continued for 6 hours. In spite of the 100 mg of Wilson's ACTH, G-2 showed less protein synthesis and no significant hormone output. In marked contrast, and even after 4 hours of perfusion with Wilson's ACTH and the apparent initial decrease in gland activity, there was a small increase in gland protein synthesis in response to Armour's ACTH in G-3 which was 1.8 times that seen in G-2. There was, in addition, during this G-3 period a simultaneous rise in hormone output indicating an effective ACTH stimulation of the cortex. Hence not only did the Armour's ACTH stimulate protein synthesis but also corticosteroid production under perfusion conditions, probably less favorable in the second experimental period than in the first.

In contrast to the above correlations and findings, our studies concerned with C^{14} incorporation into the plasma proteins were not as conclusive. Not only in AP-10, where no glands were used, but in all of the other experiments, serial plasma samples showed no significant rise in plasma protein C^{14} activity.

Table III includes the results of chromatographic analysis for hormone output. These results are in agreement with previous observations(10-12). In AP-1, AP-2, and AP-7, the hormone output in BS-2 (the perfusate during ACTH activity) was significantly higher than was the output in BS-1. Wilson's ACTH was not as effective as Armour's preparation in stimulating the cortex to secrete hormones. Thus in AP-5, there was no effect with 40 mg of Wilson's ACTH. With 100 mg in AP-6, the hormone output appeared to be only twice as much as the control sample. AP-8 represents a perfusion in which no ACTH was used; the cortical hormone output did not vary between experimental and control period. Columns 4 and 6 of Table III are included to provide a comparison between the gland activity due to protein synthesis and hormone production. The relationship has already been discussed. Column 5 is a visual comparison of the color intensities produced by the cortical hormones, of the types listed in column 7, after chromatography and treatment of the paper strips with the color-producing reagents listed above. This analysis proved adequate for a qualitative determination and as a gross index of gland activity before and after ACTH stimulation. No attempt was made to quantitate this hormone output or to identify individual compounds.

Discussion. These experiments show that ACTH, in addition to stimulating adrenal cortical hormone production, promotes protein synthesis in the isolated perfused adrenal gland. The extent to which specific enzyme proteins of the gland may be involved in the enhanced protein synthesis observed may only be conjectured. Studies of liver enzyme protein relationships have demonstrated at least a gross correlation between changes in liver protein and liver enzyme activities(13,14). Thus the effect of ACTH on adrenal gland activity may be mediated through enhanced synthesis of the enzyme proteins involved in the biosynthesis of the adrenal cortical steroids. Furthermore, this increase in protein synthesis may provide a partial explanation for the clinically and experimentally observed adrenal hypertrophy occurring *in vivo* after ACTH administration.

Our findings indicate that quantitatively varied effects are obtained with different ACTH preparations. Armour's ACTH was effective in small amounts in stimulating gland protein as well as corticosteroid synthesis. The Wilson's ACTH preparation used required larger amounts than the Armour preparation for a similar effect on protein synthesis. More striking is the lack of hormone production by the glands treated with the Wilson product used as compared with the Armour product. The relative inactivity of the former may suggest that the ACTH preparation is unstable in solution, in which form it was provided.

Summary. Isolated beef adrenal glands were perfused with homologous blood containing radioactive lysine and amino acids. One preparation of ACTH added to the perfusate was consistent in promoting gland protein synthesis in addition to an elevated corticosteroid output as compared to levels before ACTH addition. These effects were not observed with a different preparation of ACTH.

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Changes in Splenic Weight Associated with Hibernation in Bats.* (21902)

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The extreme heterothermy of bats, together with their ability to enter and leave a torpid condition rapidly and seemingly at will, has been discussed by several workers, especially Eisentraut(1). Results here presented suggest that this ability is closely associated with the storage of erythrocytes in the spleen. Pearson(4) states that "bats tend to hibernate whenever they fall asleep," their body temperature dropping to approximately that of the air. Furthermore, the diurnal rhythm of "active" bats in summer shows such great metabolic fluctuations that the maximum oxygen consumption may be as much as 18 times greater than the minimum. Such observations seem to point to the existence in bats of some kind of rapid switch mechanism, which is independent of external temperature, and which functions in turning on and off the control of body temperature.

The spleen of the ground squirrel *Citellus tridecemlineatus* contains a large amount of smooth muscle, and becomes enlarged, dark, and congested within 12 hours after the animal becomes torpid(3). Worth(5) observed that in the case of bats "winter spleens were, in general, larger than the summer spleens," but failed to recognize the significance of her observations. Evans(2) suggests that the difference in splenic volumes which he found in hibernating and active bats indicates that this organ has an important function in hibernation.

Methods. Spleens were examined and erythrocyte counts made on numerous individuals of both hibernating and active insectivorous bats (Microchiroptera). *Myotis sodalis* (Indiana bat), *Myotis lucifugus* (little brown bat), and *Eptesicus fuscus* (big brown bat) from Blackball Mine, LaSalle County, Ill., and *Tadarida brasiliensis cynocephala* (free-tailed bat) from Thomasville, Ga., were used in these experiments. Bats were maintained in a hibernating state by placing them in cigar boxes in a refrigerator at 5°C. A high humidity was insured by keeping open dishes of water in the boxes. Spleens were removed from hibernating bats immediately

TABLE I. Spleen Weights of *Myotis sodalis*.

Sex	Wt of spleen in g	Wt of bat in g	Spleen wt × 10^3/body wt
Immediately upon removal from refrigerator			
♂	.079	5.497	14.37
♂	.087	6.065	14.34
♂	.093	5.857	15.88
♀	.080	5.633	14.20
♀	.095	6.370	14.91
♀	.079	5.564	14.20
Avg	.086	5.831	14.65 ± 0.29
5 min. after being removed from refrigerator			
♂	.027	5.824	4.64
♂	.036	5.791	6.22
♂	.105	6.026	17.42
♂	.074	5.649	13.10
♂	.076	5.963	12.75
♀	.094	6.689	14.05
♀	.051	5.830	8.75
♀	.069	6.271	11.00
Avg	.066	6.005	10.99 ± 1.41
Active			
♂	.011	5.166	2.13
♂	.016	6.875	2.33
♂	.010	5.272	1.90
♂	.019	5.960	3.19
♂	.016	5.587	2.86
♂	.012	5.222	2.30
♀	.016	6.010	2.66
♀	.014	5.491	2.55
♀	.013	5.662	2.30
Avg	.014	5.694	2.47 ± 0.12

TABLE II. Spleen Weights of *Eptesicus* and *Tadarida*.

No. of individuals	Avg spleen wt in g	Avg wt of bat in g	Avg of spleen wt × 10^3/body wt
Eptesicus fuscus immediately upon removal from refrigerator			
6	.318	17.166	18.28 ± 1.00
E. fuscus when active			
7	.081	15.794	5.12 ± .65
Tadarida brasiliensis immediately upon removal from refrigerator			
4	.156	9.328	16.79 ± 2.63
T. brasiliensis when active			
4	.075	9.320	8.13 ± 2.13

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* The writers wish to thank the Department of Physiology of the University of Illinois for the facilities and equipment used in this project and Drs. John D. Anderson and C. Ladd Prosser of that Department for their advice and for help with the manuscript.

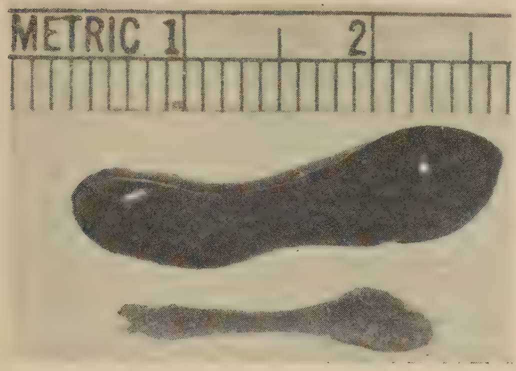

FIG. 1. Spleens of hibernating (above) and active (below) *Myotis lucifugus*.

upon disturbance. Active bats were anesthetized with ether before their spleens were removed. Spleens and bats were weighed to .1 mg. Blood samples were obtained by heart puncture with a syringe lightly heparinized by filling only the needle and base of the tube with heparin and then ejecting it completely. Sodium citrate was not a satisfactory anticoagulant for blood from active bats. Several erythrocyte counts were made on each individual.

Results. Data on spleen weights of *Myotis sodalis* are shown in Table I. Spleens from active bats weighed, on the average, 14 mg (2.47 ± 0.12 g/kg of body weight), whereas those of hibernating individuals averaged 85 mg (14.65 ± 0.29 g/kg of body weight). This represents a 500% increase in the spleen weight of these bats upon entering hibernation. The spleens of 8 hibernating individuals were examined 5 minutes after being removed from the refrigerator and placed at room temperature. The spleens of 2 had decreased greatly in size, whereas 4 had decreased only slightly, and 2 appeared not to have decreased at all, thus suggesting that there is considerable individual variation in the speed at which these bats can evacuate blood from their spleens. It may well be, however, that the initial disturbance was not sufficient to initiate the process in some individuals.

Spleens of several bats were exposed and watched as the bats became active. In one instance a male *Myotis sodalis* was taken from the refrigerator and its spleen exposed. After 6 minutes its spleen was small and completely pale, although not yet so small as that of an active bat. On another occasion, a female *Eptesicus fuscus* was taken from hibernation and its spleen exposed and observed. Initially the spleen was extremely large and dark. After 6 minutes the spleen was considerably smaller and seemed similar to some of the spleens previously taken from active bats. Nine minutes after disturbance the spleen weighed only 56 mg (3.76 g/kg of body weight). Another *Eptesicus* showed a complete evacuation of the spleen within 5 minutes.

Data on erythrocyte counts for *Myotis sodalis* are shown in Table III. The average count for 8 active bats was 13,150,000/mm^3 (range 10,610,000 to 14,380,000). Seven hibernating individuals had an average erythrocyte count of 9,340,000/mm^3 (range 7,930,000 to 11,230,000). This represents a decrease of only 41%, whereas the spleen weights had increased 500%, thus suggesting that some whole blood, as well as corpuscles, may be stored in the spleen. This would mean that the circulating blood volume in hibernating bats is somewhat less than that of active ones.

Table II shows the differences between the weights of spleens of active and hibernating individuals of the other species studied. In *Eptesicus fuscus* the spleens of hibernating individuals were about 3 times as large as those of active ones. This change is not so great as that which was found in *M. sodalis*. Spleens remained moderately large and engorged, even in the most active individuals. The difference seems to be a result of an incomplete evacuation of the spleen in *Eptesicus*. In hibernating individuals of *Eptesicus* the weight of the spleen in proportion to the

TABLE III. Erythrocyte Counts ($\times 10^{-4}$).

Hibernating		Active	
Sex	Cells/mm^3	Sex	Cells/mm^3
♂	859	♂	1353
♂	793	♂	1437
♂	908	♂	1387
♂	963	♂	1327
♀	898	♂	1061
♀	994	♀	1323
♀	1123	♀	1438
		—	1194
Avg	934	Avg	1315

body weight is even greater than in the small *Myotis*.

Tadarida brasiliensis cynocephala belongs to a semitropical family (Molossidae) and would seldom if ever have occasion to hibernate in its natural environment. After individuals of this species were induced to hibernate at 5°C, it was found that their spleens were quite large, although there was considerable individual variation. The average weight of the spleens of 4 active *Tadarida* was about one-half that of 4 hibernating ones. As in *Eptesicus*, the spleens of active individuals seemed somewhat engorged, and there was an even greater range of variation than in the hibernating ones. Three *Tadarida* were placed in a refrigerator at 0°C in order to observe the reactions of these bats to this low temperature. They were removed after 4 hours at which time they showed no visible movement or other signs of life. The spleens of 2 were much engorged with blood; that of the third had ruptured. After the spleens were removed, all 3 bats were found to be alive. *Eptesicus* kept at 0°C did not differ in their behavior from those kept at somewhat higher temperatures, *i.e.*, they were sensitive to disturbance; moved, and emitted squeaks.

Conclusions. An important factor concerned with the mechanism of hibernation in bats is the storage of erythrocytes and possibly whole blood in the spleen while in the hibernating state. Furthermore, it is believed that the rapidity with which a bat can empty its spleen is closely associated with its ability to come rapidly out from deep hibernation, as well as from various degrees of "daytime sleep." This mass release of cells from the spleen may be important in increasing the oxygen supply to the tissues, and may also be one of the factors stimulating the heart to greater activity (through the Bainbridge reflex). Investigations are continuing in an effort to establish the importance of these splenic changes in the deactivating process, as well as to establish the relationships of hormonal and possibly nervous factors which may be involved.

Summary. Data are presented which show that, in several species of bats, erythrocytes are stored in the spleen during hibernation and these are rapidly returned to circulation as the bats become active. This storage accounts for an increase in splenic weight of 500% in *Myotis sodalis*. Erythrocyte counts are lower in hibernating bats than in active ones.

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Capillary Permeability During Hemorrhagic Shock in the Rat. (21903)

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The bulk of experimental evidence strongly supports the view that a generalized increase in capillary permeability in regions remote from injury is not an important contributory element in shock resulting from hemorrhage and trauma. However, evidence does exist that such a generalized increase in capillary permeability occurs in the terminal stage of shock and may be related to the establishment of the irreversible shock state. Thus, Fine and Seligman(1) used the migration of proteins, labeled with radioactive iodine, as indicators of changes in capillary permeability. Upon intravenous injection they found a marked increase in the transcapillary passage of these proteins and their accumulation in the tissues of animals in irreversible hemorrhagic shock. Animals that survived the hemorrhagic procedure did not show such an accumulation. Wiggers and Ingraham(2) demonstrated that in animals that subsequently died in irreversible hemorrhagic shock

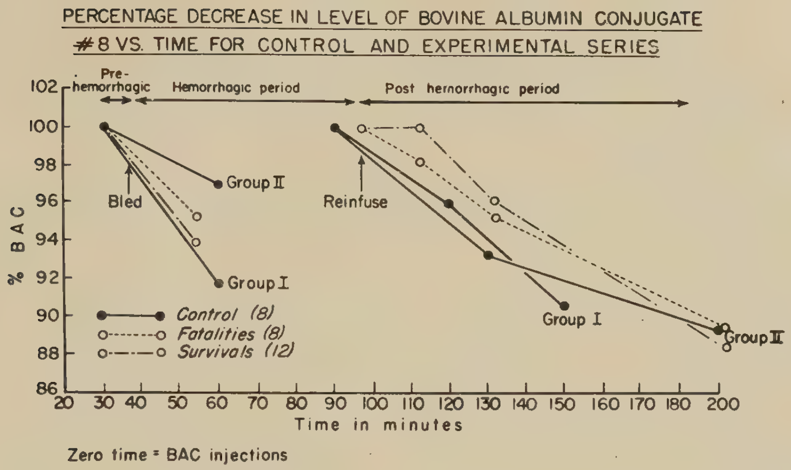

FIG. 1.

after reinfusion with whole blood, there was a progressive increase in the specific gravity of the animals' blood during the survival period to values above control levels. On the other hand animals surviving this procedure showed no such post reinfusion hemoconcentration. This suggests that an increased permeability is related to the existence of irreversibility. The recent development and use of a fluorescein labeled albumin conjugate (BAC) affords a sensitive method of following the possible transcapillary passage of albumin in shocked animals. The conjugate has physical characteristics identical to native albumin and behaves *in vivo* like its native precursor(3). It can accurately be determined quantitatively in blood and tissues in very small amounts by the methods of fluorophotometry.

Using this improved labeled protein, it should be possible to follow quantitatively small changes in capillary permeability during all stages of hemorrhagic shock from initiation to the development of irreversibility.

Method. Albino rats were subjected to various degrees of hemorrhagic hypotension, until a level and duration of hypotension was found at which approximately 50% of the animals survived. Serial bleeding to 65-70 mm Hg for 15 minutes and then to 50 mm Hg for 45 minutes resulted in a state of hemorrhagic shock in which 8 animals of a series of 20 became irreversible and died after reinfusion. Blood pressure was recorded from a cannula in the common carotid artery which was in turn connected to a strain gauge and the necessary recording instruments. A 3-way stopcock controlled the bleedings, reinfusions, and necessary blood sampling. The simple process of loosening the cannula at its junction with the stopcock and allowing 2 drops of blood to fall onto a watch glass provided ample blood for the determination of fluorescence as well as specific gravity. Using the above mentioned level and duration of hypotension, rats were injected with the fluorescein labeled albumin conjugate 30 minutes prior to bleeding. All injections were made into the saphenous branch of the femoral vein except in the Group I controls in which case the carotid artery was used. The concentration of BAC in the vascular tree was determined at various intervals and from this data time-disappearance curves were plotted. Control animals were treated in the same way except they were not hemorrhaged.

Results. The percentage decrease in the level of bovine albumin conjugate is shown in Fig. 1. The original concentration of the conjugate in the vascular tree was arbitrarily taken as 100% and the amount in the samples taken during the hypotensive period was expressed as the percentage decrease from this level. Immediately after reinfusion, a blood sample was taken, and this level of BAC again taken as 100%. The succeeding samples taken in the post-hypotensive period were

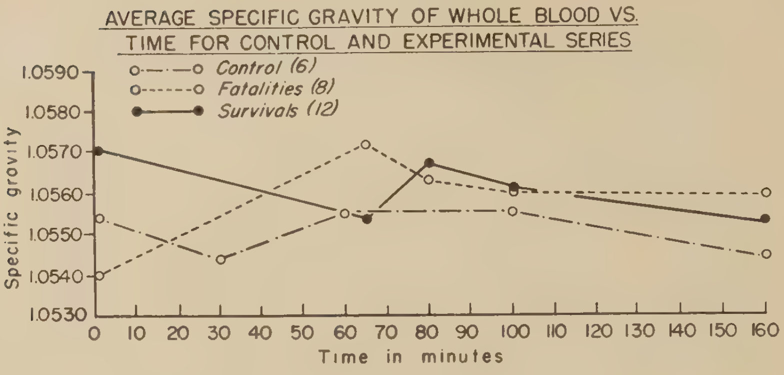

FIG. 2.

plotted as percentage decrease from this new level. The difference between the slopes of the disappearance curves for the control and experimental series appeared to be minimal and by an analysis of covariance were proven to be statistically insignificant.

Specific gravity determinations by the falling drop method showed no definite pattern of hemoconcentration or hemodilution and thus supported the fluorescein disappearance data in demonstration that no permeability change had occurred (Fig. 2).

Since the probability of an increase in permeability of the capillary bed was eliminated, tissues were not examined for accumulation of BAC. However, microscopic examination of tissues of representative animals showed no pathologic changes which would lead to the belief that the animals died of complicating factors other than hemorrhagic shock.

Discussion. The results of this investigation show there is no difference in the disappearance curves of bovine albumin conjugate from the vascular tree of control animals or those surviving or succumbing to the hemorrhagic procedures. This evidence seems to indicate that an increase in capillary permeability is not a major factor in the transition from the reversible to the irreversible phase of hemorrhagic shock in the rat.

However, as mentioned earlier, dogs in the irreversible phase showed a marked tendency toward hemoconcentration which is not seen in animals surviving the hemorrhage. The fact that no evidence of hemoconcentration was noted in rats succumbing to hemorrhage, as compared to surviving animals, seems to indicate that although the final cause of death in both species may have been the same, the mechanism appears to be different.

Whatever the sequence of events which lead to the ultimate development or irreversibility, it seems reasonably certain the final cause of death involves the loss of vasomotor tone of the peripheral vessels and consequent stasis of blood.

Summary. Under the conditions presented, in the rat, an increase in capillary permeability does not appear to be an important factor governing the transition from the reversible to the irreversible phase of hemorrhagic shock.

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Effect of Hyaluronidase and Partially Depolymerized Hyaluronate on Threshold Doses of Drugs.[*] (21904)

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The absorption of aqueous fluids injected subcutaneously is facilitated by hyaluronidase (1-3) and partially depolymerized hyaluronate(4). Harris and Cohen(5) reported that hyaluronidase enhanced the absorption of subcutaneously injected atropine as measured by changes in pupillary diameter, but noted that a method with greater sensitivity was necessary to obtain more decisive data. Our studies indicated that the antagonism by atropine of pilocarpine induced salivation was a more critical technic. Since the elapsed time between the injection of atropine and the manifestation of its action was shortened by hyaluronidase it was of interest to determine whether the induction time of a potent local anesthetic applied to the cornea could also be shortened by hyaluronidase.

Methods. Atropine vs. Pilocarpine. Studies were carried out on albino rabbits weighing 2.0 to 2.5 kg. Each rabbit was used for a maximum of 3 times at weekly intervals. One hundred and ten rabbits were tested for response to 5 mg pilocarpine nitrate/kg intravenously one week before use in the experiment. Twenty rabbits did not respond within the 3 min time limit and were discarded. The tests were conducted as follows: pilocarpine, 5 mg/kg, was administered intravenously and the animals were observed until salivation at the rate of approximately 5 ml/min occurred. One minute later the atropine was administered subcutaneously either in 1.5 ml physiological salt solution (PSS), 1.5 ml PSS containing 150 TRU hyaluronidase[†] (H), or 1.5 ml PSS containing 50 mg partially depolymerized hyaluronate (PDHA). The latter was prepared by incubating streptococcal hyaluronate with hyaluronidase for 15 min (4). Six rabbits were used for each individual dose and treatment studied. In order to evaluate the results a dose response curve for intravenous atropine sulfate was established. *Effect on corneal anesthesia.* Wy-806, whose chemistry and pharmacology will be reported later, is a glycine amide prepared from mephentermine. It is a potent local anesthetic when applied to the rabbit cornea. Anesthesia was preceded by an induction period (interval between application of the drug and manifestation of its action), a property not found by us for cocaine, procaine, Xylocaine® or Nupercaine®. After instillation of 0.2 ml 10^{-5} M Wy-806 at pH 7.2 into the conjunctival sac of rabbits the cornea was stroked with a glass fiber to determine the presence or absence of the blink reflex. Simultaneous testing in each rabbit was accomplished by instilling the local anesthetic into the left eye and the local anesthetic containing H into the right eye.

Results. Atropine vs. Pilocarpine. The average onset of salivation in 90 of the 110 rabbits receiving 5 mg pilocarpine nitrate/kg intravenously was 1-2 min (S.D. ± 0.5) and the duration of salivation was 61 min (S.D. ± 18.3). The induction period in the remaining rabbits was greater than the mean plus 2 standard deviations which was the basis for discarding them. Table I shows that the most rapid antagonism of salivation occurred with large intravenous doses of atropine. Decreasing doses of atropine resulted in a corresponding delay in the onset of action. The

TABLE I. Effect of Intravenous Atropine Sulfate on Pilocarpine Induced Salivation.

Mg atropine SO_4/kg	Inhibition by atropine SO_4 Induction period (min.) avg 6 rabbits
1.0	1
.50	2
.25	3
.125	7
.060	9
.030	19
.010	30

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[*] Presented at Fall Meeting, Am. Soc. for Pharmacol. & Exp. Therap., Charlottesville, Va., Sept. 5-7, 1954.

[†] Bovine testicular hyaluronidase assaying 7000 TRU/mg N - Wyeth Laboratories.

TABLE II. Effect of Hyaluronidase and Partially Depolymerized Hyaluronate on Atropine Antagonism to Pilocarpine Induced Salivation.

Mg atropine SO_4/kg subcut	Inhibition by atropine SO_4 Induction period (min.) avg 6 rabbits		
	PSS	+ 150 TRU hyaluronidase	+ 50 mg PDHA
3.0	1.5	—	—
2.0	3	2	2
1.0	5	2	2
.9	12	5	2
.75	17	5	2
.50	30+	5	2
.25	30+	9	7
.125	30+	30+	30+

threshold dose was considered to be 0.06 mg/kg. At this dose the onset was 9 min; longer delays overlapped on the mean duration of pilocarpine action at the lower limit (2 S.D.). Table II shows that the minimal effective dose of atropine by subcutaneous injection was reduced from 0.75 mg/kg to 0.25 mg/kg when either H or PDHA was incorporated into the injection. Smaller doses of atropine were not considered effective because they have induction periods within the range of dissipation of pilocarpine action. Fig. 1 shows that the curves are parallel and indicate a quantitative difference only. Analysis of variance by the method of Emmens(6) indicated a significant difference between intravenous and subcutaneous routes ($P>0.05$; $F = 29.8$). This difference was abolished by H and PDHA ($P<0.05$; $F = 174.3$, and $P<0.01$; $F = 297.0$ respectively). *Effect on corneal anesthesia.* All rabbits tested responded with anesthesia when $1x10^{-5}$ M Wy-806 was applied but $2x10^{-6}$ M was ineffective. Further refinement was not attempted because of the length of the induction period and it was assumed that $1x10^{-5}$ M represented the threshold 100. The average induction time for 10^{-5} M Wy-806 in 86 rabbits was 19 min (S. D. ± 5.7) with a duration of 20 min (S.D. ± 8.0). Significant decrease of induction time by H was not observed until the concentration was 360 TRU/ml (Table III). At these concentrations H had no effect on duration of anesthesia. With concentrations greater than 1100 TRU/ml anesthesia was absent during the 30 min of observation, whereas the control eye receiving local anesthetic alone was anesthetized. None of the concentrations of H used induced instantaneous anesthesia. Fig. 2 shows that 360 TRU H/ml of anesthetic solution also reduced the induction time of higher concentrations of Wy-806 without affecting the duration of anesthesia.

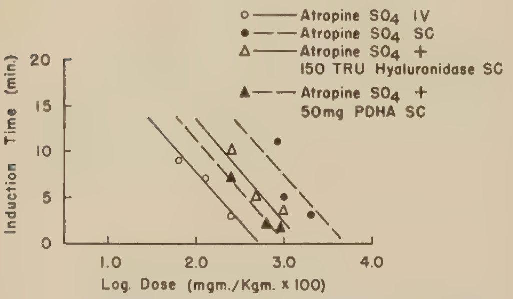

FIG. 1. Shortening of induction period of subcutaneous atropine sulfate by hyaluronidase or PDHA.

TABLE III. Effect of Hyaluronidase on Corneal Anesthesia by 1.0 × 10^{-5} M Wy-806.

TRU hyaluronidase/ml Wy-806	Time (min.) avg 6 rabbits Induction	Duration
—	19	20
131	19	"
262	14	"
360	9	"
850	5	"
1020	4	"
1062	2.5	"
1105	1*	"
1210	30+	

* 3/6 rabbits responded.

Discussion. In these studies either H or PDHA enhanced the absorption of atropine so that the subcutaneous route was no different from the intravenous with regard to rapidity of onset. These results are in agreement with the current concepts of hyaluronidase action. While the minimal effective subcutaneous dose was considerably decreased by H and PDHA it was still 4 times greater than the minimal effective intravenous dose. This confirms more decisively the observations of Harris and Cohen(5) and Seifter and Baeder (4). The data also confirm that hyaluronidase applied locally to the cornea enhances penetration of local anesthetics(3).

We are unable to account for the failure of hyaluronidase to affect the duration of corneal anesthesia produced by Wy-806. It is well known that hyaluronidase shortens the dura-

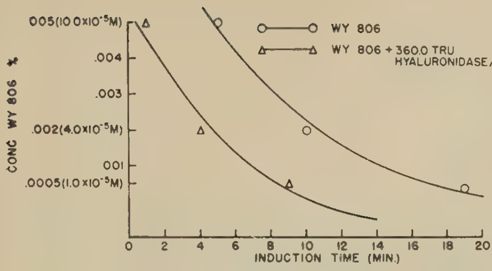

FIG. 2. Effect of hyaluronidase on induction period of varying concentrations of a local anesthetic.

tion of action of local anesthetics injected subcutaneously without epinephrine(7,8) and that the duration is inversely proportional to the concentration of the enzyme. This is usually attributed to the greater absorbing surface resulting from the spreading. The vasoconstrictor action of epinephrine decreases the rate of absorption so that with hyaluronidase it is possible to achieve a greater area of anesthesia for a given amount of local anesthetic without affecting the duration. The cornea contains the acid mucopolysaccharide hyalurono-sulfuric acid which can be depolymerized by H(9). This compound is the principal component of the ground substance of Bowman's membrane which contains the sensory nerve endings. From Bowman's membrane the ground substance extends in tubule-like structures through the stroma(10) to Descemet's membrane and all substances must pass through these before having access to the anterior chamber for absorption. It has been shown that local anesthesia is a function of nerve permeability(11) and that the duration of local anesthesia measured the time necessary for the diffusion process to decrease the concentration of drug to a threshold level(12). The induction time is a measure of the time required for penetration of solutes by diffusion through the connective tissue sheath of the nerve(13). Probably H depolymerized the ground substance of Bowman's membrane and facilitated penetration of the local anesthetic so that for a given dose a larger number of receptors became available and thereby decreased the induction time. Larger concentrations of enzyme probably caused extensive depolymerization of the ground substance with sufficiently rapid dispersion into the anterior chamber through Bowman's membrane, the stroma, and Descemet's membrane so that only subminimal concentrations of anesthetic were available.

Summary. 1. Hyaluronidase or partially depolymerized hyaluronate enhanced the absorption of subcutaneously injected atropine so that this route was no different from the intravenous with regard to rapidity of onset. 2. Hyaluronidase or partially depolymerized hyaluronate also lowered the minimal effective dose of subcutaneous atropine necessary to antagonize pilocarpine induced salivation, 3. Hyaluronidase applied topically to the cornea shortened the induction time of a local anesthetic at the threshold concentration without affecting the duration of action. Higher concentrations of hyaluronidase abolished the local anesthesia.

We wish to thank Dr. A. J. Plummer, Ciba Pharmaceutical Products, Summit, N. J. who kindly supplied the Nupercaine® used in these studies, and Mr. Edward E. Livingston, Jr., for technical assistance.

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Morphological Changes in the Growing Rat Skull Following Administration of Cortisone Acetate.[*] (21905)

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Retardation of skeletal maturation in the growing rat follows the administration of cortisone, Parmer *et al.*(1); Follis(2). Several visceral components of the rat skull exhibit diverse behavior under similar experimental conditions; the growth and differentiation of teeth and alveolar bone are stimulated, Goldsmith *et al.*(3,4), while that of the brain is partially suppressed, Field(5).

The osseous facial and neural components of the skull, largely reflecting the morphology of the protected or supported cranial viscera Starck(6), have no *necessary* morphological correlation with each other, Hofer(7). As the cranial bones and viscera exhibit a differential response following cortisone therapy, it should be possible to display experimentally the morphogenic role of these cranial viscera. This paper presents the data of such a demonstration.

Methods. Thirty Long-Evans rats were subcutaneously injected with 50 γ/g body weight of cortisone acetate on the day of birth (first day), and on the second, fourth and sixth days. These rats and 35 controls were sacrificed on the fifteenth day. Intact skulls were prepared by boiling in water and bleaching in 3% H_2O_2.

The skull vault dimensions measured were: the length of the internasal, metopic, sagittal, coronal, anterior lambdoid and tempero-parietal sutures, the total skull length and the greatest skull width. The detailed technic was reported by Moss(8). The occlusal length of the mandibular molar arcade and the height of the ramus (coronoidangular processes) were also measured. The greatest length and width of the cerebrum were measured, *in situ,* by the technic of Sugita(9), following removal of the calvaria.

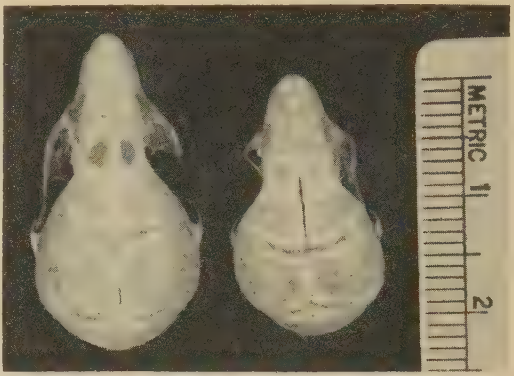

FIG. 1. Fifteen-day rat skulls, normal at left, cortisone-treated at right, showing gross size differential. Note especially lack of interdigitation of treated rats sagittal suture and hypodevelopment of lambdoid region in this animal.

Results. The effect of the preparation on vault dimensions was tested and proved to be negligible (Table III). All experimental bone dimensions were significantly smaller than controls ($p<.01$) (Table I, Fig. 1). The dimensional reduction of the skull vault as a whole was not proportional. The facial vault exhibited greater reduction than the neural vault. In terms of percentage, the order of magnitude of the reduction of facial skull vault length (dimension 5), height of ramus (dimension 9) and body weight (dimension 13) were similar; and as a group, greater than those of the neural skull vault. In addition to this regional disproportionality, within the neural vault itself the reduction of the cerebral and skull vault dimensions were also disproportionate, producing a paralleled "brachycephaly" (Table II, index #1). Calculation of cephalic indices further demonstrated the relatively greater reduction of the visceral skull dimensions (Table II, index #2).

The mandibular molar teeth were unaffected by the drug. They exhibited a normal occlusal length (dimension 10) and crown pattern, and were well embedded in

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[*] Cortisone acetate supplied by Merck and Co. Inc., Rahway, N. J.

[†] Work done under the tenure of a Lederle Medical Faculty Award.

TABLE I. Dimensional Values of 15-day-old Rats Following Administration of Cortisone Acetate.

Description	Exper. mean (mm)	N	Control (mm)	N	% reduction
Coronal suture	5.7 ± .10*	19	6.2 ± .07	16	8
Sagittal "	4.8 ± .07	19	5.9 ± .11	16	18
Ant. lambdoid suture	5.6 ± .11	19	6.2 ± .16	16	9
Tempero-parietal suture	9.0 ± .20	19	10.3 ± .18	16	13
Internasal suture	6.3 ± .11	19	8.4 ± .23	16	25
Lambda-anterior nasal point	19.6 ± .15	19	23.4 ± .26	16	16
Metopic suture	8.2 ± .10	19	9.2 ± .14	16	11
Squamosal width	12.2 ± .08	19	13.6 ± .26	16	10
Coronoid-angular processes	5.7 ± .07	10	6.9 ± .04	10	17
Length of molar arcade	6.6 ± .04	10	6.6 ± .03	10	0
Parasagittal length of cerebrum	11.4 ± .23	11	12.3 ± .15	19	7
Greatest width of cerebrum	12.7 ± .16	11	13.1 ± .09	19	3
Body wt (g)	18.8 ± .58	11	23.5 ± .98	19	20

* Standard error of mean calculated for small samples.

TABLE II. Cephalic Indices of Normal and Experimental Rats.

Index No.	Dimensions (× 100)	Exp.	Control
1	$\frac{\text{Squamosal width (8)}}{\text{Metopic suture (7) + sagittal suture (2)}}$	93.8	90.0
2	$\frac{\text{Squamosal width (8)}}{\text{Lambda-anterior nasal point (6)}}$	62.2	54.7
3	$\frac{\text{Cerebral length (11)}}{\text{Cerebral width (12)}}$	90.0	94.0

TABLE III. Effect of Preparation on Skeletal Vault Dimensions.

	Mean shrinkage in mm	
Dimension	(Exp.) N = 6	(Control) N = 8
Squamosal width (8)	.2	.5
Internasal suture length (5)	.0	.1
Lambda-anterior nasal point (6)	.6	.9

alveolar bone, confirming and extending the findings of Goldsmith *et al.*(3,4). The molar arcade, as a whole, was more posteriorly situated relative to the mandibular foramen. The areas of muscle attachment, coronoid and angular processes (dimension 9), were significantly reduced ($p < .01$).

Histological sections of the vault revealed deficient growth of cortical plates, diploe and sutural areas, as compared with the controls (Moss, 8). The sutural tissues were underdeveloped, relatively acellular, avascular and more fibrous. At sacrifice, the vault bones were freely movable in the treated rats, but not in the controls. This mobility was apparently related to the immature state of the bone edges at the sutural areas, *i.e.*, their lack of interdigitation.

Discussion. The administration of cortisone to neonatal rats is followed by disproportionate morphological changes in the growing membranous skull bones. No evidence presently available indicates intrinsically different responses to such therapy in adjacent skull vault bones. The relatively greater retardation of the facial skull vault compared with the neural vault implies that, under these experimental conditions, brain size and shape are competent morphogenic agents.

The growing cerebrum, experimentally retarded to a lesser degree, is capable of overcoming a greater degree of osseous retardation. It causes the formation of a sufficient area of vault to provide for its own protection. This is not accomplished by merely separating smaller bones and increasing the width of the sutures. In some manner osteogenesis is stimulated, although only immature bone is produced in the experimental rats. The parallel brachycephalization of cerebrum and skull vault supports this statement. Disproportionate cerebral dimensions apparently evoke similar osseous disproportions. Examination of the data of Moss(8) and of Sugita (9) clearly shows that the absolute dimensions and relative proportions of the experimental rats are *not* those of younger rats. There is no normal developmental stage at which all of these osseous and neural dimensions occur simultaneously, absolutely or relatively.

The hypothesis that the morphology of skull components are directly related to the size and function of their related viscera(van der Klaauw, 10) is further supported by the mandibular data. The dimensionally normal molar dentition of the experimental rats is provided with adequate alveolar bone, despite the generalized reduction of the basal mandibular bone.

Decreased facial vault length implies a growth retardation (25%) of the respiratory components of the facial skull approximating that experienced by the body as a whole (20%). The retardation of nasal bone length cannot be attributed to the effect on the cartilage found at the anterior nasal margin. It has been shown that the chief growth site, in length, of the facial vault is at the frontonasal sutural area(Massler and Schour, 11). The percent of nasal bone length retardation is identical with that reported by Mortimer (12) (25%), following hypophysectomy of young adult rats.

The retardation of body weight and linear dimensional values may be due to either a general inanition response to the drug, or to a specific effect of cortisone. In this experiment, cortisone was used only as a tool. The results obtained are, in this sense, independent of the mechanisms of their production.

Summary. 1. Disproportionate changes in skull and brain morphology followed the subcutaneous administration of 50 γ/g of cortisone to neonatal rats. 2. The visceral skull vault showed greater retardation than the neural skull vault. 3. The cerebrum and neural skull vault also showed disproportionate dimensional changes leading to increasing brachycephalization. 4. The growing rat brain and molar teeth are competent morphogenic agents. Under these experimental conditions, they are capable of overcoming the relatively greater retardation of related bone, and of thus providing for their own functional protection and support.

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Cobalt-Induced Polycythemia and Survival of X-Irradiated Albino Rats.*† (21906)

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Because the development of anemia is one of the effects of whole-body irradiation, any factors which stimulate the production of erythrocytes and hemoglobin are of interest. Smith *et al.*(1) used a simulated high altitude to induce a polycythemia in rats, and found that these altitude-acclimatized rats had poorer survival after whole-body x-irradiation than did similarly irradiated control rats. A marked increase in the number of erythrocytes and in the concentration of hemoglobin can also be produced in rats by the administration of cobalt salts(2). Constant and Phillips(3) reported that the long-term feeding of a polycythemic quantity of cobalt in a natural diet increased the rate of mortality of rats which received whole-body irradiation. Parr *et al.*(4) reported that mice fed a diet containing an unknown amount of cobalt chloride for a short period (5 to 8 days) prior to irradiation and 15 days after, showed a significant increase in resistance to the effects of irradiation. These same investigators(5), however, subsequently found that the administration of a cobalt-containing diet to mice for 36 days before and 15 days after irradiation produced no beneficial effects. Korol and Coon(6), who also fed an unknown amount of dietary cobalt chloride to mice for 5 days before and 17 days after irradiation, found that the cobalt had no effect on the ultimate 28-day mortality of the irradiated mice, but that it did cause a slight increase in the ST_{50} (survival time for 50% of the animals).

In the present study, we have observed the effects of various dietary concentrations of ionic cobalt on the increased production of hemoglobin, and the effects of increased hemoglobin concentrations on the survival of rats after whole-body irradiation. Mucoprotein concentrations in plasma were also measured for some rats in each group at 3 and 2 weeks before irradiation, and at 48 hours after.

Experimental. Sixty male and 60 female Sprague-Dawley rats, 28 days old, were equally distributed by weight and sex into 6 experimental groups, after they had been fed a basal, purified diet for one week. To facilitate the handling of rats during the determinations of hemoglobin and mucoprotein and during irradiation, each group was composed of 2 subgroups which were started on experiment 3 weeks apart. The 6 groups were given, *ad libitum,* distilled water and the basal diet plus the amounts of cobalt shown in Table I.

The basal diet had the following percentage composition: casein, 20; L-cystine, 0.2; Salts IV‡(7), 4; cottonseed oil, 6; dextrin, 11; sucrose, 58.7; choline chloride, 0.1; and vitamins. The vitamins were included in the following amounts in mg/kg of diet: thiamine•HCl, 3; pyridoxine•HCl, 2.5; riboflavin, 3; niacin, 15; calcium pantothenate, 20; biotin, 0.1; folic acid, 0.2; vit. B_{12}, 0.01; menadione, 4; and inositol, 100. Vit. A was added, as Nopco stabilized A, to provide 5000 I U/kg of diet; vit. D was added, as irradiated yeast, to provide 1000 I U/kg diet. In addition, Haliver oil was given intermittently by dropper to insure an adequate supply of vit. A. Cobaltous chloride was included in the diets of Groups II through VI as a mix which was prepared by dispersing and drying a solution of 20.19 g $CoCl_2 \cdot 6H_2O$ in ground dextrin to total 5000 g. For each 10 ppm Co desired, 10 g of carrier/kg diet were used.

Hemoglobin concentrations were deter-

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‡ The Salts IV also included a cobalt salt to provide 1 ppm Co in the diet.

TABLE I. Relationship between Dietary Cobalt and Increases in Hemoglobin.

Exp. group No.	I	II	III	IV	V	VI
Ppm added dietary Co	0	10	20	40	70	100
Increase in Hb (14 wk) (g/100 ml blood)	3.7	4.4	5.6	7.1	8.2	8.8
$R = \frac{\text{Incr. Hb in exp. group}}{\text{Incr. Hb in Group I}}$	1.0	1.19	1.51	1.92	2.22	2.38
Logarithm (ppm added Co)	—	1.00	1.30	1.60	1.84	2.00
$K = \frac{R}{\text{Log (ppm added Co)}}$	—	1.19	1.16	1.20	1.20	1.19

mined for all rats at 28 days of age and at intervals thereafter, by the usual, oxyhemoglobin, colorimetric method, on blood from the tail veins.

After 16 weeks on the experimental diets, again after 17 weeks, and for a third time at 48 hours after irradiation, the concentration of mucoprotein (measured as mucoprotein-tyrosine) in the plasma was determined on about half of the rats in each group. A modification of the method of Winzler *et al.*(8) was used, and the rats were fasted 4 to 6 hours before 1.5 to 2 ml of blood were obtained from each by heart puncture, under brief, ether anesthesia.

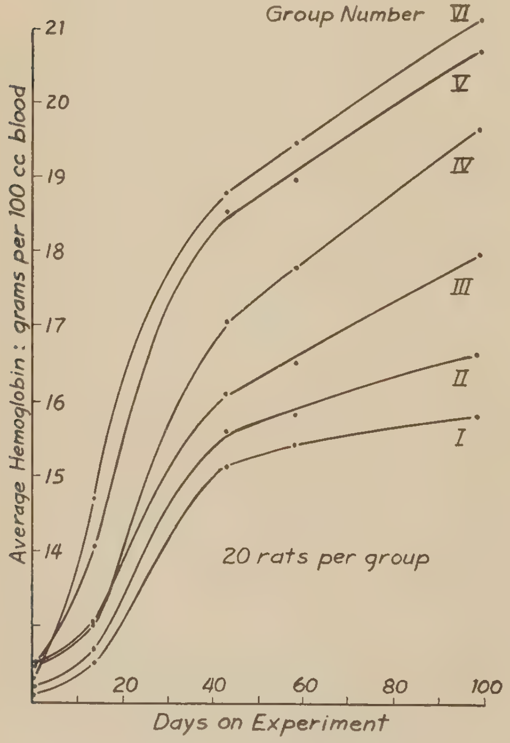

FIG. 1. Increases in hemoglobin concentrations.

After 19 weeks on the experimental diets, all rats were exposed, in groups of 8 to 13, to 750 roentgens whole-body x-irradiation, delivered by a Westinghouse unit at 14.5 r/min. The radiation factors were: 175 KV, 10 ma, 0.5 mm Cu and 1 mm Al added filtration, and 60 cm target-specimen distance.

Results. The course of the average production of hemoglobin by rats which received the basal diet and each cobalt-containing diet is shown, for the period before the withdrawals of blood by heart puncture, in Fig. 1. The numerical relationship between the concentration of added dietary cobalt and the production of excess hemoglobin in 14 weeks is shown in Table I. The ratio of the increase in the concentration of hemoglobin in 14 weeks in rats which received added cobalt, to the normal increase in hemoglobin in the rats which received the basal diet, is directly proportional to the logarithm of the concentration (expressed in ppm) of added dietary cobalt.

The average rate of growth of male rats was slightly decreased in the group which received 100 ppm of cobalt, whereas that of the females was slightly decreased in the groups which received 70 and 100 ppm.

The average concentrations of mucoprotein-tyrosine in the plasmas of rats in the 6 groups varied from 6.8 to 9.6 mg/100 ml before irradiation, and from 8.2 to 11.2 after irradiation, but the differences between groups and the changes at 48 hours after irradiation were not shown to be significant.

At the time of irradiation, there were 16 to 18 rats, instead of 20, in each group because

TABLE II. Survival after 750 r Whole-Body X-Irradiation.

Exp. group No.	I	II	III	IV	V	VI
No blood withdrawn:						
Fraction surviving*	4/10	4/8	1/9	3/9	1/9	2/9
Avg† time (days)	24.4	25.4	14.1	18.0	13.4	15.6
ST_{50}‡	28.5	35+	13	11.5	12	11
Blood withdrawn 3 times:						
Fraction surviving	1/6	1/8	0/8	3/7	1/9	2/8
Avg time (days)	17.2	15.6	8.6	19.7	15.1	24.6
ST_{50}	18.5	11	8	9	13.5	28.5
All rats in each group:						
Fraction surviving	5/16	5/16	1/17	6/16	2/18	4/17
Avg time (days)	21.6	20.5	11.5	18.7	14.2	19.8
ST_{50}	20.5	15.5	10	11.5	12.5	15

* No. rats surviving beyond 35 days/No. rats in group.

† All rats which survived beyond 35 days were counted as having a 35-day survival in all average survival times.

‡ Survival time (in days) for 50% or more of the group.

a few had been lost prior to that, through accidental deaths. Since some rats in each group underwent the stress of the withdrawal of blood on 2 occasions before irradiation and once after, the measurements on survival were made separately for those rats which did and did not have blood taken from them. The figures on survival are presented in Table II. Of the rats that did not have any blood withdrawn, those which received 20 ppm or more of cobalt in the diet had poorer survival than those which were fed no added cobalt or only 10 ppm. The withdrawals of blood seemed to have the greatest deleterious effects in these latter 2 groups, Nos. I and II. In the groups which were fed 40 and 70 ppm of cobalt, the bleedings by heart puncture had no apparent influence on survival; but in the group which received 100 ppm, the blood withdrawals tended to increase the average survival time. When all of the rats are taken into consideration, it is concluded that the addition of cobalt to the diet certainly had no beneficial effect on survival after irradiation; it appears, instead, to have decreased the time of survival.

Discussion. The development of various degrees of polycythemia in rats, by the addition of known quantities of cobalt to a purified diet, was apparently not hindered by the presence of 0.2% L-cystine in the diet. Orent and Bucciero(9) reported that cysteine effectively inhibited the development of polycythemia by cobalt, whereas methionine showed no such effect, even though it caused an improvement in growth. The effect of cystine seemed to resemble that of methionine, rather than that of cysteine. Growth was good in all groups in the present study, although it was slightly inhibited at the higher concentrations of dietary cobalt. The amounts of cobalt-intake which decreased the rate of growth were about the same as those which were found to be toxic for the rat by Stare and Elvehjem(10).

The greater concentrations of hemoglobin in the blood of cobalt-fed rats were of no benefit, but rather a hindrance, for increasing either the percent survival or the average length of survival times. These results are in agreement with the observation by Constant and Phillips(3). They also confirm the results that Smith *et al.*(1) obtained with rats in which a polycythemia was induced by repeated exposures to reduced pressure. If dietary cobalt is capable of increasing the resistance of rats to the lethal effects of whole-body irradiation, as reported by Parr *et al.* (4) for mice after short-term feeding, it is not due to the polycythemia-inducing action of cobalt.

For the rats in Group VI, the withdrawals of blood apparently helped to increase their average survival time. Since some rats in this group had hematocrit values of 65 to 70 at the time of the first heart punctures, possibly a decrease in the viscosity of the blood, brought about by the loss of blood, was a factor in increasing the survival time.

The measuring of mucoprotein in the plasma was of interest because the authors previously found (unpublished data) that the concentration of mucoprotein in the plasma of dogs often increased several fold just before death from x-irradiation.

Summary. Cobaltous chloride was incorporated in the diet of rats in the amounts of 0, 10, 20, 40, 70 and 100 ppm of added cobalt for a period of 19 weeks prior to whole-body x-irradiation and 6 weeks after. The various degrees of polycythemia which were thereby induced were measured indirectly by measuring the increases beyond the normal in hemoglobin concentrations. The ratio of the increase in hemoglobin concentration in each polycythemic group to the normal increase in 14 weeks was found to be directly proportional to the logarithm of the concentration (expressed in ppm) of added dietary cobalt. The rats with cobalt-induced higher concentrations of hemoglobin had an increased average rate of mortality after receiving 750 r whole-body x-irradiation, rather than a decreased rate, as compared with similarly irradiated control rats. Measurements of mucoprotein concentrations in the plasma revealed no significant differences between the various groups before irradiation, nor substantial changes at 48 hours after irradiation.

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Isolation and Cytopathogenic Effect of Influenza B Viruses in Monkey Kidney Cultures.* (21907)

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(Introduced by Laurence W. Roth.)

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The primary isolation of influenza A and B viruses in cultures of human embryonic lung or kidney has been described(1,2). That cultures of monkey kidney might be a suitable substitute for human tissues was suggested by the similarities in behaviour and characteristics of these viruses in either type of culture (3,4). Thus, titers of hemagglutinating and infectious virus were equivalent and cytopathogenic effects were similar in infected cultures of cells from both species(3). Hemagglutinating activity, too, was not altered by passage to monkey kidney cultures. Influenza A agglutinated human type O erythrocytes at a higher titer than fowl erythrocytes at 4°C, while influenza B agglutinated both types of erythrocytes to the same titer(4).

In this communication it is shown that isolations of influenza B viruses were obtained more frequently and with greater facility in cultures of monkey kidney than in the amni-

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TABLE I. Isolation of Influenza B Viruses in Cultures of Monkey Kidney and in Amniotic Sac of Chick Embryo.

Nasal washing No.	Monkey kidney cultures				Chick embryo isolation on passage No.
	Hemagglutination titer[*]		Cytopathogenic effect[†]		
	3 day	6 day	3 day	6 day	
1954					
1701	6	18	0	2+	1
1736	0	656	0	3+	1
1739	0	400	0	3+	1
1741	40	9	1+	2+	1
1746	32	30	0	2+	1
1756	36	153	1+	2+	1
1768	6	113	0	2+	2
1771	0	41	0	2+	2
1779	24	240	1+	2+	1
1811	14	5	0	1+	1
1845	5	72	1+	2+	1
1899	48	35	2+	2+	1
1909	0	177	0	2+	2
1912	0	65	0	2+	‡
1954	29	5	0	1+	1
1955					
2231	0	160	0	3+	1
2234	0	8	0	1+	2
2235	0	32	0	2+	1
2237	2	52	1+	2+	1
2239	0	53	0	2+	1
2240	0	132	0	3+	‡
Controls	0	0	0	0	0

[*] Arithmetic mean of titers with 0.125% human type O erythrocytes at 4°C. Titers less than 4 recorded as 0.

[†] Cells affected: 1+ 25%, 2+ 50%, 3+ 75%.

[‡] Not isolated on third passage.

otic sac of the chick embryo, and the probable mechanisms of these differences are described. The cytopathogenic effect accompanying growth of influenza viruses on initial isolation is also demonstrated.

Methods. Kidneys from adult male Rhesus monkeys were minced with scissors and digested for 2 to 3 hours with 1.0 mg/ml crystallized trypsin in Hanks' solution(5) at 37°C until the material passed readily through the tip of a 10 ml serological pipette. After 3 washings the digested kidney tissue was suspended in 30 ml of Hanks' solution and 0.1 ml was pipetted into 20x150 mm Pyrex tubes. The suspension was spread over the inner surface of the tubes which were placed in a horizontal position for one hour prior to addition of 2.0 ml of nutrient medium: bovine amniotic fluid, 90%, bovine embryo extract, 5%, inactivated horse serum, 5%, phenol red, 0.002%, penicillin, 100 units per ml and streptomycin, 100 γ/ml(6). The cultures were then rolled at 12 rev/hour at 35°C. Epithelial cell growth covering approximately one-half of the planted surface was attained in 10-14 days. Nutrient medium was replaced twice weekly during this period. Only cultures with multiplying cells and with little evidence of degeneration were used in experiments. Nasal washings from military personnel with acute respiratory infections were collected in the spring of 1954 and the winter of 1955 in 20 ml of beef-heart infusion broth or phosphate buffered saline. Immediately after collection inactivated horse serum was added to a concentration of 10%. The specimens were refrigerated for 1 to 2 hours, mixed in a cold blendor for 2 minutes, and then stored in sealed ampoules at -60°C. Each specimen was inoculated into 4-6 monkey kidney cultures in 0.1 ml amounts and also into the amniotic sac of 10-11 day chick embryos by means of a closed technic(7). Amniotic fluids were harvested at 4 days and tissue culture

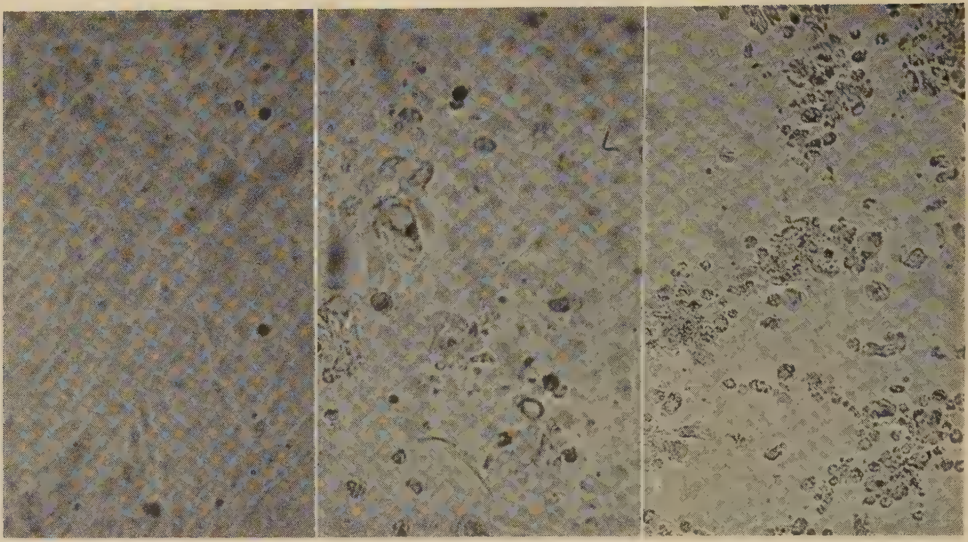
Control culture Early degeneration Advanced degeneration

FIG. 1. Cytopathogenic effects of influenza B virus multiplication in monkey kidney culture epithelial cells. Photographs of unstained roller tube cultures (× 78).

fluids at 3 and 6 days. Hemagglutination titrations were done at 4°C by the pattern method with human type O or pooled fowl erythrocytes in a final concentration of 0.125%. Negative amniotic fluids were inoculated into a second group of chick embryos. Virus infectivity titers (ID_{50}) were determined by inoculating 0.1 ml of 10-fold dilutions of virus into the amniotic sac and incubating 4 days or into monkey kidney cultures and incubating 7 days(3). Fluids were titrated for hemagglutinins and the end points (EID_{50} or $MKID_{50}$) determined by the method of Reed and Muench(8). Hemagglutination-inhibition titrations were performed with influenza virus-immune rooster antisera and acute and convalescent human sera treated with receptor-destroying enzyme prepared from the Inaba strain of Vibrio cholera(9). Immune sera to chick embryo adapted influenza A and B strains, GL 1148-52A and GL 1760-54B, were prepared in roosters and heated at 56°C for 30 minutes for virus neutralization in tissue cultures.

Results. Primary isolation: Influenza B viruses which were isolated from nasal washings are shown in Table I. A single passage was sufficient in monkey kidney cultures for virus to be detected by hemagglutination in every instance. The mean titer shown in some cases was low in both the third and sixth day fluids due to lack of viral multiplication in some cultures which were probably inoculated with specimens containing virus at the end-point of infectivity. In the amniotic sac of the chick embryo a second passage was necessary in order to detect hemagglutinins with 4 of the strains inoculated, and in 2 others hemagglutinins were not present after 3 passages. Both the tissue culture and chick embryo lines of each virus shown were identified as influenza B by hemagglutination-inhibition with a rooster antiserum prepared against a chick embryo adapted 1954 strain. Inhibition titers were similar regardless of the host system in which the virus had been propagated.

Cytopathogenic effects. Also shown in Table I was the degree of cytopathogenic effect in cultures of monkey kidney which accompanied growth of virus. There was close agreement between the titer of hemagglutinins in the culture fluids and the amount of cytopathogenic changes in the cells. With the technics employed in these experiments in which living cultures were examined microscopically, it was not possible to detect cytopathogenic changes before virus had multi-

TABLE II. Infectivity and Hemagglutination Titers of Pooled Tissue Culture Fluids Following Inoculation with Nasal Washings Containing Influenza B Virus.

		Reciprocal logarithm of dilution		
			Tissue culture fluids	
Specimen	Method of titration	Nasal washing	3 day	6 day
1736	HA*	<.6	<.6	2.82
	EID_{50}	1.33	2.0	3.5
	$MKID_{50}$	—	4.33	4.12
2239	HA*	<.6	<.6	1.72
	EID_{50}	.75	2.4	2.88
	$MKID_{50}$	2.33	5.0	3.3

* Hemagglutination titer with human type O erythrocytes, 0.125%.

plied sufficiently for hemagglutinins to be titrated. Even when hemagglutinin titers were lower in the second tissue culture fluid, cell damage was usually greater than that found at the time of the first fluid changes. However, total cell destruction was not observed. Fig. 1a, b, c, demonstrate the progressive changes from an uninoculated culture to a culture with extensive cell destruction. Initially there was granularity of the cytoplasm with swelling and rounding of the involved cell. Later the nucleus appeared granular and shrunken. Clumps of rounded cells were seen at first on the periphery of the cellular outgrowth but later were scattered throughout. Advanced stages revealed pyknotic and fragmented cells with debris scattered about the culture.

Growth characteristics. Increase in infectious virus as well as hemagglutinins was demonstrated in cultures inoculated with influenza B. Titers of infectivity were determined both in the amniotic sac of 10-11-day chick embryos and in cultures of monkey kidney (Table II). The results were uniformly higher in the latter system indicating that more viral particles were required for infection of the amniotic sac than for infection of monkey kidney cultures. Hemagglutinating titers were highest in the fluids removed on the 6th day. A considerable amount of this virus was probably non-infectious as suggested by the high Ha/ID_{50} ratio at that time(10).

Neutralization. Viral multiplication and cytopathogenic effects were not demonstrated when influenza B antiserum was added to the specimen prior to inoculation of cultures. In Table III are shown the results of such an experiment in which rooster antiserum against a 1954 strain of influenza B was added to nasal washings containing infectious virus. Following the first change in nutrient medium without additional antiserum viral multiplication took place as determined by the appearance of hemagglutinins and cytopathogenic effects. Since the neutralizing antiserum had been prepared with a twelfth chick embryo passage of influenza B it is unlikely that antibody to another virus was present in significant quantity. Neutralization of the cytopathogenic changes by this antiserum therefore supplied additional evidence that they were due to influenza B virus.

Attempts to isolate influenza viruses not infectious for the chick embryo. Nasal washings from 18 men which had been collected within 24 hours of the onset of an acute febrile respiratory infection during the influenza outbreak were selected for study. From none of these specimens had influenza virus been isolated by 2 passages in the chick embryo. These men all had at least a 4-fold rise in antibody to influenza B as determined by hemagglutination-inhibition. Inoculation of nasal washings into the amniotic sac of the chick embryo as well as cultures of monkey kidney resulted in virus isolation in 2 instances (Table I) in the latter system, whereas hemagglutinins were not found even after 3 passages in the chick embryo. Both of these strains multiplied readily in the amniotic sac upon inoculation of the virus propagated in monkey kidney cultures.

Discussion. Although the number of additional influenza B isolations which were obtained by the use of monkey kidney cultures was small, this may have been influenced con-

TABLE III. Neutralization of Influenza Virus Growth in Monkey Kidney Cultures by Specific Rooster Antiserum.

Inoculum	Influenza antiserum	Hemagglutination titer* 3 day	Hemagglutination titer* 6 day	Cytopathogenic effect† 3 day	Cytopathogenic effect† 6 day
Nasal washing	None	2	48	1+	2+
No. 1845	B‡	0	6	0	1+
(Log EID_{50} <1.0)	A	8	96	1+	2+
Nasal washing	None	24	40	2+	2+
No. 1899	B‡	0	48	0	2+
(Log EID_{50}, 1.4)	A	48	33	2+	2+
Controls	None	0	0	0	0

* Arithmetic mean of titers with 0.125% human type O erythrocytes at 4°C. Titers less than 4 recorded as 0.

† Cells affected: 1+ 25%, 2+ 50%.

‡ *In ovo* neutralization titer, 1/25.4.

siderably by the plan of the study. Had more specimens been inoculated into these cultures it is quite likely that a greater difference would have been demonstrated when the results were compared to those from the chick embryo. Evidence for this could be found in the growth characteristics of these unadapted strains in monkey kidney. (1) Second passages were unnecessary in the latter but were frequently required for virus isolation in the amniotic sac. This may have been due to the presence of antibody from the nasal secretions(11) which would not have inhibited viral multiplication after changes in nutrient fluids in tissue cultures. In the chick embryo, however, another passage might have been necessary for sufficient dilution of the antibody. (2) In the order of 10 to 100 times as many viral particles were required for infection of the chick embryo as were required for infection of cultures of monkey kidney. The difficulties of technics of inoculation and harvesting of amniotic fluids might be considered another factor in comparing the relative efficacy of these two systems for the isolation of influenza B viruses.

Time allowed for growth of viruses was longer in cultures of monkey kidney than in the chick embryo. It had been previously demonstrated(3) that influenza B propagated in cultures of human lung required 4 days for the smallest infectious inocula to multiply to maximum titer in the amniotic sac of the chick embryo. A strain isolated and propagated in monkey kidney required 6 days in the latter cultures(12). Therefore, in these experiments adequate growth time was allowed in both systems, and a longer period would not have shown additional viral increments in either.

The occurrence of cytopathogenic effects on initial isolation in monkey kidney cells was like that observed following growth of influenza viruses in human embryonic kidney and lung(3) suggesting similarities in the cells from these species. This behaviour was not manifest when one of these strains was propagated in cultures of chick embryo lung(12). Moreover, only after several passages in the amniotic sac were these strains definitely lethal for the chick embryo(12). Analogous findings have been reported for the effects of unadapted influenza virus in the mouse lung in which maximum growth occurs, but pneumonia was not demonstrable unless the virus were adapted to the mouse(13). Thus, it follows that influenza viruses may be adapted to growth in human or monkey renal cells even on initial isolation.

Summary. Influenza B viruses obtained in 1954 and 1955 have been isolated directly in cultures of monkey kidney. The latter system was shown to be preferable to the amniotic sac of the chick embryo for this purpose since more viruses were isolated and only single passages were necessary. Factors have been described which serve to explain the mechanism of these advantages. Growth of these strains was accompanied by cytopathogenic effects in the epithelial cells of monkey kidney cultures.

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Ventricular Excitability During Hypothermia and Rewarming in the Dog.* (21908)

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Considerable attention has been devoted to the general subject of hypothermia and its effect on myocardial irritability. The appearance of ventricular arrhythmias at and below a body temperature of 25°C has fostered the assumption that the excitability of the mammalian ventricle increases as the body temperature falls. Direct measurements of ventricular excitability in dogs subjected to immersion hypothermia supported this hypothesis(1). The present study describes the changes in excitability which the ventricular myocardium exhibits not only during cooling to 25°C but also during rewarming, with and without artificial ventilation for blood pH control.

Methods. The experimental data presented herein were accumulated on 7 dogs ranging in weight from 8.5-13.4 kg, and anesthetized with pentobarbital sodium (30 mg/kg I.P.). Electrocardiographic recordings (Lead II) were taken on all dogs following attachment of standard limb leads. Thoracotomy at the left fourth intercostal space was performed and 2 Ag•AgCl electrodes placed on the heart, one on the left auricle and the second on the left ventricle. The auricular electrode served as a pacemaker for the heart while the ventricular electrode was used to measure the threshold of the left ventricle at all points in its cycle. Following electrode attachment the chest was closed and pneumothorax was reduced. Five dogs were allowed to respire spontaneously throughout the cooling and rewarming procedure. Two dogs were artificially respired in order to maintain a near-normal pH during hypothermia.

The technic employed to measure ventricular threshold was essentially as previously described(2). The range of current strength at our disposal was increased in this study by placement of a cathode ray oscilloscope in the testing circuit between the ventricular stimulator and the heart. In this way the current applied to the heart could be measured directly by means of the deflection of the cathode ray beam on the calibrated oscilloscope screen. This modification increased the maximum current strength of the circuit from 2.73 to approximately 30 ma.

Left intraventricular pressure was recorded by placement of a #8 radio-opaque catheter in the left ventricle via the common carotid artery in order to determine whether the ectopic electrical responses produced by the application of test stimuli to the ventricle possessed a mechanical component as well.

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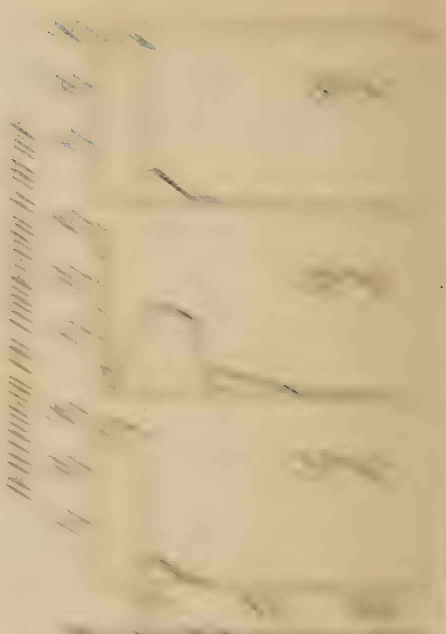

temperature (mean pulse rate, 150) to 412 msec. at 25° (mean pulse rate, 62). The QRS interval, *i.e.*, ventricular conduction time, also increased from a mean of 38 msec. to 60 msec. Both the total "refractory" period and the ventricular conduction time reverted to normal during rewarming.

Discussion. Although hypothermia does exert a general depressant action on bodily functions, its effect on the excitability of the ventricle is one of stimulation. The evidence presented here and elsewhere(1,2,4) confirms the hypothesis that ventricular arrhythmias at low body temperatures indicate an increased myocardial irritability. These and other recent studies indicate that the cause of this augmented excitability is the acidosis which accompanies the fall in temperature(4). The intracellular changes which ultimately are responsible for the observed alterations in ventricular threshold are obviously of a reversible nature since rewarming restores the threshold to pre-cooling levels. The exact nature of these cellular changes is not known, but more recent work(5) indicates that the mechanisms controlling electrolyte balances between blood and myocardium are affected.

The extremely low threshold early in systole observed at low body temperature and pH raises the question of what is meant by cardiac refractoriness. Obviously at this point in the excitability cycle of the hypothermic ventricle, the myocardium is neither absolutely nor relatively refractory. Therefore, in view of the data presented some revision in our terminology with respect to the phases of excitability through which the heart passes appears necessary.

Summary. All portions of the excitability cycle of the dog's ventricle suffer a progressive fall in threshold as body temperature is lowered to 25° under conditions of spontaneous respiration. The decrease in threshold is especially marked in early systole at a point designated as the minor dip. This general increase in myocardial irritability is a reversible phenomenon, the normal excitability recovery curve being reestablished by rewarming to normal body temperature. However, no change in ventricular excitability except at the major dip obtains during hypothermia provided the pH of the blood is maintained at a normal level throughout the cooling process. Both the total "refractory" period and the ventricular conduction time are prolonged during cooling and return to normal during rewarming.

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Urinary and Plasma Amino Nitrogen in Irradiated Dogs.* (21909)

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Aminoaciduria has been observed in persons exposed to lethal and sublethal doses of ionizing radiation(1,2). The increased urinary excretion of amino acids, maximum on the sixth to eighth post-exposure day and not proportional to the dose, has also been noted in a human subject exposed to less than 15 reps of total body radiation(2). A preliminary report also indicates that the plasma amino acid level in dogs is doubled after irradiation with x-rays(3).

In the present experiments, the plasma and

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urinary amino nitrogen was studied in dogs subjected to a lethal dose of x-rays. Since food intake influences amino acid metabolism, non-irradiated control dogs pair-fed with the experimental animals were studied simultaneously.

Methods. In the first experiment, 3 experimental male and 3 control female dogs of comparable weight (8-10 kg) were housed individually in metabolism cages. They were given water *ad libitum* and were fed Purina dog chow. After irradiation, the diet of all animals was restricted so that the food intake of the test and control animals was equal. During the control pre-irradiation study period of 7 days, 24-hour urine samples were collected over thymol and stored at 4°C. Three to 4 ml of blood were taken daily before feeding and a protein-free solution of the serum was prepared immediately according to the method of Somogyi(4). These samples were also stored in the cold over thymol. On the day before irradiation and on the 3 days following, blood was drawn 3 times daily: 9:00 a. m. (fasting), 3:00 p. m. (shortly after feeding) and 9:00 p. m. Collection of urine was continued for 5 days after exposure and then every second day until the ninth post-exposure day.

In the second experiment, a single male dog was studied before and after exposure to a supralethal dose of x-rays. The dog was allowed to eat and drink *ad libitum* and samples were collected in the manner just described.

The radiation dose given to the experimental dogs in the first experiment was 450 roentgens (an estimated L. D. 50/30 days). Seven hundred roentgens (an L. D. 100/30 days) was given to the single dog in the second experiment. The radiation factors were: 250 k.v. peak voltage, 15 ma, aluminum parabolic filter and 0.5 mm copper, 40 inches skin-target distance and a radiation intensity of 7.4-7.8 roentgens/min. The quantitative ninhydrin method of Moore and Stein(5) was adapted for analysis of the total amino nitrogen content of the samples. Each sample was assayed in triplicate and the results were expressed in terms of a leucine standard which would give an equivalent reading. Before the method could be applied to urine, it was necessary to remove all traces of ammonia and this was accomplished by bubbling nitrogen for one hour through alkalinized, diluted urine samples placed in boiling water. One-tenth to two-tenths ml of the treated diluted urine brought to a pH 5 with dilute HCl was used for all determinations.

TABLE I. Total Urinary Amino Nitrogen (mM Leucine Equivalent/24 Hr).

Time after radiation, days	450 roentgens exposure			700 roentgens exposure (1 dog)
	Avg control (3 dogs)	Avg exp. (3 dogs)	Difference	
–6	15.6	15.7	– .1	
–5	12.8	15.1	– 2.3	
–4	19.6	11.2	+8.4	27.10
–3	13.5	13.5	0	17.3
–2	15.5	16.5	– 1.0	13.9
–1	14.3	8.1	+6.2	20.0
Avg and stand. dev.			1.9 ± 1.8	19.57
+1	17.2	7.8	+9.4	16.8
+2	10.8	7.5	+3.3	9.6
+3	7.5	18.0	–10.5	5.4
+4	10.8	21.0	–10.2	8.7
+5	17.3	14.1	+3.2	27.6
+6	15.3	7.9	+7.4	17.2
+8	12.1	25.4	–13.3	16.7
+10	6.3	6.6	– .3	14.6
Avg and stand. dev.			–1.4 ± 1.4	14.57

Descending paper chromatography according to the method of Consden, Gordon and Martin(6) was applied to 7 selected urine specimens showing the lowest pre-exposure amino nitrogen and the highest post-exposure levels. With the solvent employed, *i.e.* a 1:1 mixture of commercially available collidine and lutidine (saturated with water), the amino acids moved slowly on the paper and this often resulted in poor separation of the spots. Better separation was obtained by first running the 2 dimensional chromatograms in the recommended way and then drying the papers and re-running them with the collidine-lutidine solvent. Finally, the papers were dried and sprayed with 0.5% ninhydrin solution in 95% ethanol.

Results. Tables I and II show the comparative values for urine and plasma amino nitrogen in irradiated and control dogs of the first experiment. Occasional high values are found in samples from irradiated and control dogs with about equal frequency. The average daily difference (control group average

TABLE II. Plasma Amino Nitrogen (mM Leucine Equivalent/Liter Plasma).

	450 roentgens exposure			700 roentgens exposure (1 dog)
Time after radiation, days	Avg control (3 dogs)	Avg exp. (3 dogs)	Difference	
–4	5.1	9.3	– 4.2	3.2
–1	3.1	3.3	– 0.2	3.6
	Avg and stand. dev.		–2.2 ± 2.0	3.40
+ 1	3.5	3.0	+ .5	4.1
+ 2	3.8	2.9	+ .9	4.2
+ 3	2.9	3.6	– .7	3.8
+ 4	5.6	5.0	+ .6	2.6
+ 5	9.9	4.1	+5.8	2.9
+ 6	3.5	3.5	0	3.2
+ 8	4.6	4.0	+ .6	
+10	3.3	3.8	– .5	
	Avg and stand. dev.		.9 ± 1.0	3.35

minus irradiated group average) in urinary amino nitrogen was 1.9 ± 1.8 mM leucine equivalent/day. The standard deviation of a daily difference between the output of the 3 controls and the 3 irradiated dogs was 4.3 mM leucine equivalent/day in the pre-exposure period. Thus, a single difference would have to be 11.2 mM leucine equivalent/day to be considered significant at the 5% probability level. Only one daily difference (8th day post-irradiation) was found to exceed this value. As was seen by examining the output of the individual animals, this large average difference is due entirely to the high output of one irradiated dog. The average post-exposure difference was –1.4 ± 1.4 indicating an empirical but not significant increase in the daily amino nitrogen output of irradiated animals.

Similar treatment of the data on plasma amino nitrogen shows a significant increase in the plasma amino nitrogen level in the control dogs on the fifth post-exposure day. This is unquestionably due to a real increase in the control values rather than a relative decrease in the irradiated dogs since each plasma level in individual control dogs increased on this day. The average daily pre-irradiation difference (control average minus irradiated average) in total plasma amino nitrogen was –2.2 ± 2.0 mM leucine equivalent/L. and the same value for the post-irradiation period 0.9 ± 1.0.

In the second experiment, no change was observed in the urinary plasma or urinary amino nitrogen levels of the single dog given a supralethal dose of x-rays. It is concluded, therefore, that our data do not indicate a real change in either the urinary or plasma amino nitrogen in these animals following irradiation under the conditions of this experiment.

The following amino acids were identified on the paper chromatograms: glycine, serine, alanine, valine, leucine, theonine, histidine, glutamic acid, aspartic acid, taurine, arginine and lysine. Visual examination disclosed no obvious changes in the type or relative concentration of the amino acid components in urine samples studied before and after irradiation.

Discussion. Hall and Whipple(7) observed more than 30 years ago an increased excretion of nitrogen after irradiation of the abdominal region of dogs. Prosser and co-workers(8) confirmed this observation. They demonstrated that the nitrogen balance of dogs exposed to total body x-radiation was temporarily negative. Irradiated rats and their pair-fed controls are also found to be in negative nitrogen balance during the first 6 days after exposure or reduction of food intake(9). The nitrogen loss of the irradiated animals was about 30% greater than that of their controls. In view of these observations and others concerned with disturbed amino acid metabolism in rodents(10,11), it is surprising that a disturbance in plasma and urinary amino acids similar to that occurring in man was not observed in the present study. Since the number of animals used was small, we cannot conclude with certainty that dogs and men show a different pattern of blood and urinary amino nitrogen after irradiation; nevertheless, a species difference seems possible.

Summary. The plasma and urinary amino nitrogen content was studied in dogs exposed to an L. D. 50 (and pair-fed non-irradiated controls) and to an L. D. 100 of x-rays. No significant abnormalities were noted in contrast to the pronounced aminoaciduria reported to occur in man exposed even to small doses of total body radiation.

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Incorporation of $C^{14}O_2$ into Organic Compounds by Cell-free Extracts of Autotrophically Grown Hydrogenomonas sp.* (21910)

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The ability of heterotrophic tissue to fix carbon dioxide has been well established(2) and intensive investigations on the pathways of the carbon of carbon dioxide in photosynthesis have been reported(3). However, in the case of the autotrophic bacteria, which like photosynthetic organisms are capable of utilizing carbon dioxide as the sole source of carbon, little is known about the mechanism or mechanisms by which they assimilate carbon dioxide. In view of the widespread occurrence of certain fixation reactions in heterotrophic organisms it would not be unexpected to find the same reactions operative also in the autotrophic bacteria.

The test organism studied was a species of Hydrogenomonas which is capable of growing autotrophically by utilizing the energy provided by the oxidation of hydrogen gas, or heterotrophically on a number of organic compounds. The existence of some of the known mechanisms by which carbon dioxide is utilized was investigated by examining cell-free extracts of autotrophically grown cells of Hydrogenomonas for their ability to perform several of the beta-carboxylation reactions.

Materials and methods. The strain of Hydrogenomonas used was obtained from Prof. P. W. Wilson, and was identified by Schatz and Bovell(4) as *Hydrogenomonas facilis.* Stock cultures and mass cultures were made on the following medium (in g): $MgSO_4 \cdot 7H_2O$, 0.2; KH_2PO_4, 1.0; NaCl, 0.2; $NaHCO_3$, 0.7; NH_4Cl, 1.0; Fe^{+++} (as $FeCl_3$), 0.1 ppm; and distilled water, to 1000 ml. For stock slants, 2% washed agar was added. Mass culturing of the organism was done in an apparatus consisting of a 14 liter growth bottle (with 8 liters of medium) connected to a closed system through which was circulated the gas mixture consisting of (at atmospheric pressure) 5 to 10% oxygen, 10% carbon dioxide, 30 to 60% hydrogen, and the remainder nitrogen. This composition was maintained during growth by the addition of fresh gas mixture as needed. Heterotrophically grown cells were obtained on the above medium which contained also 2 g per liter of lactic acid (neutralized with KOH), under an

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atmosphere consisting of 40% air, 5 to 10% carbon dioxide, and 50 to 55% nitrogen (the gas mixture was replenished after 6, 18 and 30 hours of incubation). The periods of incubation for the heterotrophic and autotrophic cultures were 40 hours, and from 4 to 5 days at 25°C, respectively. The inoculum for either type of culture consisted of 2 flask cultures (100 ml of the medium for autotrophic growth in a 500 ml flask), which had been incubated under the mixture of hydrogen, oxygen, and carbon dioxide for 48 hours.

Cells were harvested in a Sharples centrifuge and washed once with distilled water. Cell-free extracts were prepared in a Raytheon 9 kc. sonic disintegrator, and the supernatant liquid after centrifugation at approximately 15,000 x g constituted the crude cell-free extract. Experiments involving incorporation of C^{14} from $NaHC^{14}O_3$ into organic compounds were performed in 15 ml capacity Warburg flasks with no alkali in the center well. At the end of the desired period of incubation, the flask contents were added to 2.5 ml of 10% metaphosphoric acid, aerated with nitrogen gas for 5 minutes to remove the remaining $C^{14}O_2$, and the precipitated protein was removed by centrifugation. The total non-volatile radioactivity[‡] fixed into the reaction mixtures was estimated by plating an aliquot of the latter (after metaphosphoric acid treatment), and counting the radioactivity in a gas-flow windowless counter. Suitable corrections were made for self absorption. The activity in the beta-carboxyl of oxalacetic acid was determined by decarboxylation with Al^{+++} as described by Krebs and Eggleston(5). In all cases, the counts were statistically treated to obtain the "nine-tenths" error(6).

To locate the activity in specific compounds, the acidified reaction mixtures were extracted with ether according to the method of Neish(7), and the concentrated ether extracts were examined by descending chromatography with the benzyl formate solvent system of Lugg and Overell(8), as modified by Goldschmidt(9) in the following manner: the solvent system contained 100 ml of benzyl alcohol, 100 ml of distilled water, and 64 ml of 90% formic acid. The mixture was shaken periodically until 2 waterclear layers resulted (3 to 7 days), and the lower layer was used as the mobile phase. To locate the acids on the paper the chromatograms were sprayed with a 0.04% solution of bromocresol green in ethyl alcohol (with enough NaOH added to give a distinct blue color). The chromatograms were placed on Kodak 14 inch by 17 inch no-screen X-ray film to locate the radioactive spots. The exposure time was from 15 to 30 days, depending on the amount of activity on the chromatogram.

Oxalacetic acid was converted to its 2, 4-dinitrophenyl-hydrazone derivative essentially by the method of Cavallini, *et al.*(10). This derivative was chromatographed ascendingly with the solvent system of Warburton, *et al.*(11).

Results. Incorporation of label from $NaHC^{14}O_3$ into malic acid. The Hydrogenomonas extracts were examined for the ability to incorporate $C^{14}O_2$ into malic acid (with $NaHC^{14}O_3$ as the source of $C^{14}O_2$). A reaction mixture containing cell-free extract, DPNH, $NaHC^{14}O_3$, pyruvic acid, and Mn^{++} ions was incubated, and it was found that radioactivity was incorporated into the organic components of the reaction mixture. TPNH could be substituted for DPNH. In the exchange reaction where malic acid plus TPN or DPN were substituted for the reduced forms of the 2 coenzymes, a similar lack of specificity for coenzyme was observed (Tables I and II). Mn^{++} ions and coenzyme both are needed for optimum incorporation of radioactivity in

TABLE I. Fixation of Label from $NaHC^{14}O_3$ by a Cell-Free Extract of Hydrogenomonas in Presence of Reduced DPN and Pyruvic Acid.

Additions	Total counts fixed (cpm)
3.5 μM DPN	435 ± 313
4.7 μM DPNH	7900 ± 547

Reaction mixture contained the following: Pyruvic acid, 370 μM; $MnCl_2$, 8 μM; sodium ATP, 3.5 μM; carrier $NaHCO_3$, 480 μM; $NaHC^{14}O_3$, 1.7 × 10^6 cpm; enzyme preparation, 0.2 ml. Total volume, 3.2 ml with distilled water. Gas phase, 50% N_2 and 50% CO_2. Reaction time, 3.5 hr at 30°C. Organic acids were neutralized with $KHCO_3$ before use in all experiments.

[‡] Volatile compounds such as formic and acetic acids may have been lost in part during the process of removing the $C^{14}O_2$ at the end of the reaction.

TABLE II. Fixation of Label from $NaHC^{14}O_3$ by a Cell-Free Extract of Hydrogenomonas in Presence of Pyruvic Acid, Malic Acid, and DPN.

Additions	Total counts fixed (cpm)
Complete	43,600 ± 945
–$MnCl_2$	5,580 ± 424
–Pyruvic acid	34,600 ± 938
–DPN	1,190 ± 355
Heat-killed enzyme	638 ± 289

Reaction mixture contained the following: Pyruvic acid, 185 μM; $MnCl_2$, 8 μM; DPN, 0.75 μM; L-malic acid, 20 μM; sodium ATP, 3.5 μM; carrier $NaHCO_3$, 240 μM; $NaHC^{14}O_3$, 6.7 × 10^6 cpm; enzyme preparation, 0.15 ml. Total volume, 3.0 ml with distilled water. Gas phase, 50% N_2 and 50% CO_2. Reaction time, 4 hr at 30°C.

this exchange reaction. It would be necessary to purify the extracts in order to determine whether the requirement for coenzyme is truly non-specific. All of the radioactivity of these reaction mixtures was ether soluble; paper chromatography and corresponding autoradiograms indicated that essentially all of the radioactivity was in malic acid.

The mechanism by which malic acid becomes labeled in the presence of radioactive bicarbonate and pyruvic acid should be interpreted cautiously. The experimental conditions suggest that "malic enzyme" system is operative but it is also possible that malic acid is being labeled by some alternative or unknown mechanism.

Incorporation of label from $NaHC^{14}O_3$ into oxalacetic acid. The presence of an enzyme in Hydrogenomonas extracts capable of incorporating radioactive carbon dioxide into oxalacetic acid was determined by the general technic of Krampitz and Werkman(12), in which the enzyme is permitted to decarboxylate oxalacetic acid in the presence of labeled bicarbonate. Any exchange occurring simultaneously and resulting in the labeling of oxalacetic acid can be determined by counting the activity in the carbon dioxide obtained by decarboxylation with Al^{+++} ions of the remaining oxalacetic acid. The results obtained with extracts of Hydrogenomonas indicate that carbon dioxide is incorporated into the beta-carboxyl of oxalacetic acid only in the presence of ATP (Table III). ADP could be substituted for ATP. Mn^{++} ions enhance the amount of radioactivity fixed; neither DPN nor TPN seems to have an effect on the amount of C^{14} incorporated into the beta-carboxyl of oxalacetic acid. That the radioactivity actually is in oxalacetic acid was further confirmed by the treatment of the reaction mixture with 2,4-dinitrophenylhydrazine, and co-chromatography of the isolated hydrazones. The labeled spot coincided with the position of the hydrazone of oxalacetic acid on the paper chromatogram. Further evidence is given by the decrease in radioactivity of the hydrazone upon heating; this is characteristic of the lability of the oxalacetic acid hydrazone. The extracts of Hydrogenomonas are also capable of decarboxylating oxalacetic acid as was shown by measuring manometrically the release of carbon dioxide from this compound(13).

The finding that ATP is needed for the incorporation of radioactivity into oxalacetic acid in the presence of pyruvic acid, oxalacetic acid, and Mn^{++} ions agrees with the findings regarding the pigeon liver system. The role of ATP in this reaction has been discussed by Utter and his coworkers(2,14,15).

Incorporation of label from $NaHC^{14}O_3$ into isocitric acid. It has been established(13) that carbon dioxide can react with alpha-ketoglutaric acid to yield isocitric acid via oxalosuccinic acid. When preparations of Hydrogenomonas were examined for their ability to fix labeled carbon dioxide (as $NaHC^{14}O_3$) in the presence of TPNH, alpha-ketoglutaric acid, and Mn^{++} ions, it was found that ether extractable radioactive material was formed. Chromatography and autoradiography indicated that most of the

TABLE III. Fixation of C^{14} from $NaHC^{14}O_3$ by a Cell-Free Extract of Hydrogenomonas in Presence of Pyruvic and Oxalacetic Acids.

Additions	Total counts fixed in oxalacetic acid (cpm)
Complete system	16,990 ± 374
–ATP	1,406 ± 207
–$MnCl_2$	4,150 ± 223
–Pyruvic acid	10,525 ± 332
–Oxalacetic acid	1,907 ± 215

Reaction mixture contained the following: Pyruvic acid, 185 μM; oxalacetic acid, 60 μM; $MnCl_2$, 8 μM; sodium ATP, 3.5 μM; carrier $NaHCO_3$, 475 μM; $NaHC^{14}O_3$, 16.2 × 10^6 cpm; enzyme preparation, 0.35 ml. Total volume, 3.0 ml. Gas phase, 50% N_2 and 50% CO_2. Reaction time, 4 hr at 30°C.

TABLE IV. Fixation of C^{14} from $NaHC^{14}O_3$ by a Cell-Free Extract of Hydrogenomonas in Presence of Alpha-Ketoglutaric Acid, Isocitric Acid, and TPN.

Additions	Total counts fixed (cpm)
Complete system	21,000 ± 710
–TPN	9,890 ± 502
–TPN and isocitric acid	812 ± 35

Reaction mixture contained the following: Alpha-ketoglutaric acid, 100 μM; $MnCl_2$, 8 μM; TPN, 0.65 μM; $NaHCO_3$, 360 μM; $NaHC^{14}O_3$, 6.75 × 10^6 cpm; DL-isocitric acid, 29 μM; enzyme preparation, 0.3 ml. Total volume, 3.0 ml with distilled water. Gas phase, 50% N_2 and 50% CO_2. Reaction time, 4 hr at 30°C.

radioactivity was in a compound having the R_f value of isocitric acid. Radioactive carbon dioxide was incorporated also into a compound with the same R_f value when TPNH was replaced by isocitric acid and TPN (Table IV). The identity of this radioactive compound was confirmed by the use of pig heart isocitric dehydrogenase as described by Ochoa(13). The compound eluted from the chromatogram was found to be levorotatory and stable to heat; also, the pig heart isocitric dehydrogenase caused the release of radioactivity (as $C^{14}O_2$). This would indicate that the isocitric acid in the eluate was labeled in the tertiary carboxyl carbon; this atom would be expected to be labeled as a result of the reaction of $C^{14}O_2$ and alpha-ketoglutaric acid.

Incorporation of labeled carbon dioxide into organic compounds by heterotrophically grown cells. Because Hydrogenomonas is a facultative autotroph, the opportunity was provided for surveying, in a cursory manner, the potentialities of a cell-free extract from heterotrophically grown cells to perform the labeling reactions that were shown with preparations from autotrophically grown cells. The extracts of the heterotrophically grown cells incorporated radioactive carbon dioxide into malic acid, isocitric acid, and the beta-carboxyl group of oxalacetic acid under the same experimental conditions as the extracts from autotrophically grown cells.

Discussion. The data obtained with cell-free extracts from autotrophically grown cells indicate the potentiality of the test organism to utilize certain mechanisms for the fixation of carbon dioxide. On the basis of our present knowledge of comparative biochemistry one might have predicted that the enzymes for these reactions would be present in autotrophic bacteria. What remains to be demonstrated is the proportion, if any, of the total carbon dioxide fixed by these particular reactions during autotrophic growth. We are now following the fate of radioactive carbon dioxide in cells of Hydrogenomonas growing under autotrophic conditions in the presence and absence of inhibitors, and hope that this study will clarify this question.

Summary. Cell-free extracts from autotrophically grown Hydrogenomonas, in the presence of $NaHC^{14}O_3$ and certain organic acids, are capable of performing the following reactions: 1) To incorporate C^{14} into malic acid, in the presence of TPNH or DPNH, pyruvic acid, and Mn^{++} ions; the same is accomplished when malic acid plus TPN or DPN instead of the reduced forms of the coenzymes are used in the above reaction mixtures. 2) To incorporate C^{14} into the beta-carboxyl group of oxalacetic acid when $NaHC^{14}O_3$ is incubated with pyruvic acid, Mn^{++} ions, and oxalacetic acid; ATP is required for this reaction. 3) To incorporate C^{14} into isocitric acid when $NaHC^{14}O_3$ is incubated with alpha-ketoglutaric acid, Mn^{++} ions, TPNH, or with isocitric acid and TPN in place of TPNH. Cell-free extracts of heterotrophically grown Hydrogenomonas, like the extracts from autotrophically grown cells, are able to accomplish the same reactions.

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Strepogenin Activity of Synthetic Peptides Related to Oxytocin. (21911)

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Because partial hydrolysates of several proteins are necessary for optimal growth of *Lactobacillus casei, L. bulgaricus,* hemolytic streptococci, and higher animals(1-4) under certain defined conditions, the idea of a peptide growth factor, named strepogenin, has arisen. In the presence of all the known amino acids of protein origin, these organisms fail to grow normally unless this growth factor is added. There is much evidence to indicate that the active material in partial hydrolysates of proteins is a peptide, or a group of related peptides. Recently(5), this view has been much strengthened by the fact that high strepogenin activity for the growth of *L. casei* could be demonstrated in a synthetic polypeptide, oxytocin, one of the hormones of the posterior pituitary gland. The structure of oxytocin has been established synthetically(6,7) as a cyclic cystine-containing peptide as represented by I.‡

Arginine-vasopressin,§ in which phenylalanine replaces the isoleucine and arginine replaces the leucine of Structure I has been shown also to possess considerable strepogenin activity(5).

The question naturally arose as to whether the entire molecular structure of oxytocin was necessary for its strepogenin activity or whether activity could reside in some smaller fragment of the molecule, or was due possibly to some special feature of the molecule, such as the 20-membered disulfide ring. From the work on the synthesis of oxytocin, the heptapeptide amide derivative, tosyl-L-isoleucyl-L-glutaminyl - L - asparaginyl - S - benzyl-L-cysteinyl- L -prolyl-L-leucylglycinamide (II), which had been used as an intermediate, was available. We thought it was of interest to convert this protected peptide into the free peptide, *i.e.*, the heptapeptide amide disulfide, and test it for strepogenin activity. The compound (II) was therefore reduced with sodium in liquid ammonia and the resulting sulfhydryl compound was subsequently oxidized by aeration in aqueous solution. The crude reaction-product was assayed and found to have a high degree of activity. When it was assumed that the yield of free peptide from the protected peptide was quantitative, the results of assay indicated that the hepta-

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[*] With the technical assistance of V. Armbrust.

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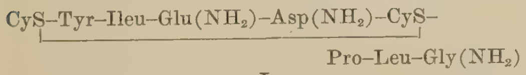
[‡] In its essential features, the nomenclature used follows the conventions of Brand and collaborators (8,9).



$$\text{CyS–Tyr–Ileu–Glu(NH}_2\text{)–Asp(NH}_2\text{)–CyS–Pro–Leu–Gly(NH}_2\text{)}$$

I

[§] The vasopressin was purified natural material isolated from beef posterior lobes; S, S′-dibenzyl oxytocin, performic acid-oxidized oxytocin, and trypsin-treated vasopressin were prepared from natural material. Oxytocin and all other peptides were synthetic.

peptide amide disulfide had a potency of 330 units per milligram, an activity not significantly different from that of oxytocin itself, which assayed 300 strepogenin units per milligram(5). Unlike oxytocin, however, the material showed negligible avian-depressor and oxytocic activities. The strepogenin-active heptapeptide amide disulfide, *i.e.,* the disulfide of L-isoleucyl-L-glutaminyl-L-asparaginyl-L -cysteinyl- L-prolyl-L -leucylglycinamide, was isolated by extraction and purified by countercurrent distribution between 0.01 *M* ammonia and *sec*-butanol. In this system it possessed the distribution coefficient ($K = 0.4$). Assay of the material in the peak tubes showed a potency of 345 strepogenin units per milligram. This activity was approximately the same as had been deduced from assaying the unpurified reaction-mixture resulting from reduction of the protected heptapeptide, followed by oxidation. Another peptide derivative, namely, S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, was also available from the synthetic work related to oxytocin. In this case, however, reduction and subsequent oxidation comparable to that used with the heptapeptide derivative led to little if any strepogenin activity. It was thought desirable to prepare a number of peptides related in structure to but simpler than the active heptapeptide disulfide and test these for strepogenin activity. Several such compounds have now been prepared which lack successive amino acid residues at either end of the molecule. These are tosyl-L-glutaminyl-L-asparaginyl-S-benzyl - L-cysteinyl-L-prolyl-L-leucylglycinamide; carbobenzoxy-L-isoglutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl - L prolyl-L-leucylglycinamide; carbobenzoxy - L asparaginyl-S -benzyl- L-cysteinyl-L- prolyl-L-leucylglycinamide; tosyl-L-isoleucyl - L-glutaminyl - L - asparaginyl- S -benzyl - L - cysteinamide; carbobenzoxy - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L-cysteinyl - L - prolinamide; and tosyl - L isoleucyl - L - glutaminyl - L - asparaginyl-S-benzyl - L - cysteinyl - L - prolyl - L - leucylglycine benzyl ester. These synthetic peptide derivatives were prepared‖ by the general methods used in the synthesis of oxytocin (6,7,10-12). The terminal amino group was protected by a tosyl (*p*-toluenesulfonyl) or carbobenzoxy group and the sulfhydryl group by the benzyl grouping. In one case a carboxyl group was protected as the benzyl ester. The peptides were obtained from these by suitable reduction with sodium in liquid ammonia, which removed all protecting groups. Assays for strepogenin activity were performed on the crude reduction products after aeration in aqueous solution to convert the resulting sulfhydryl peptides to the disulfides. Although it was realized that the activities derived from testing the crude mixtures depended upon the yields in the reductions, and therefore might be somewhat lower than the values for the isolated peptides, it was felt that, barring gross side reactions on reduction, a series of similar peptides could be scanned easily in this way and a reasonable idea of at least relative potencies could be obtained. As already described for the case of one of the most active peptides, the disulfide of Ileu-Glu (NH_2) - Asp(NH_2) - CySH - Pro-Leu-Gly (NH_2), the peptide was isolated from the crude mixture and purified, and the potency of the purified material agreed with that deduced from assay of the crude mixture.

The question arose as to whether the sulfhydryl form of the peptides differed in activity from the corresponding disulfides. In an attempt to maintain the sulfhydryl form cysteine¶ was added to solutions of the peptides obtained from the sodium-liquid ammonia reduction. No material difference in potency was found between this preparation and the disulfide.

Methods of assay. All assays for strepogenin activity were done with the aid of *L. casei* according to the directions previously published(1), as modified in(13). Wilson's

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‖ Details of the preparation of the compounds will be submitted for publication elsewhere.

¶ Cysteine did not interfere with the assay when it was present at concentrations less than 10 μg per cc. Above this concentration this amino acid alone caused growth. Quantitative assay showed that cysteine had a strepogenin potency of 1 unit per mg. Cystine had a potency of 0.3 unit per mg, even though the basal medium already contained much cystine. However, these activities were negligible compared with those of the peptides.

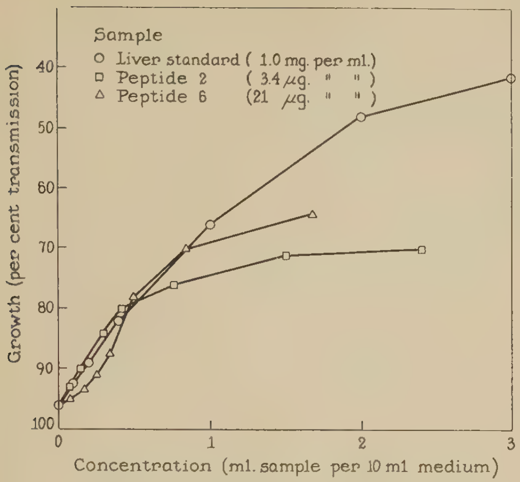

FIG. 1.

liver fraction L(1) was used as standard, and was assigned a potency of 1 unit per mg. Under the conditions of the test, half-maximal growth of the organism was produced by about 90 γ of liver extract per cc.

Peptides were dissolved in water and were added to the basal medium which was then sterilized by autoclaving. The disulfide of Glu(NH$_2$) - Asp(NH$_2$) - CySH-Pro-Leu-Gly (NH$_2$) was sterilized by filtration of its solution, since one might expect this compound to be converted to the pyrolidonecarboxylic acid derivative when heated. Actual experiment showed that inactivation did occur on autoclaving. Computation of the strepogenin potency of some of the peptides was rendered uncertain because the shape of the dose-response curves obtained with them was different than for the liver standard. In general, the active peptides did not produce maximal growth, but tended to give response curves which flattened out sooner than the standard with increasing dosage. This is illustrated in Fig. 1 in the curve for peptide 2. In addition one compound, the disulfide of Asp(NH$_2$)-CySH-Pro-Leu-Gly(NH$_2$), gave a curve of distinctly different shape. This is also shown in Fig. 1. Because of these deviations all values for strepogenin activities were calculated from dosages which gave no more than half maximal growth on the scale for liver standard.

Results and discussion. The results are summarized in Table I, where it can be seen that several smaller peptides related to oxytocin had significant strepogenin activity. From these data it was clear that with respect to this biological activity the whole molecule of oxytocin was not required. Since the activity of peptide 2 [*viz.,* the disulfide of Ileu-Glu(NH$_2$) - Asp (NH$_2$) - CySH-Pro-Leu-Gly (NH$_2$)] approached that of oxytocin itself, neither the cysteinyl--tyrosyl moiety nor the cyclic disulfide structure in oxytocin appeared to be necessary for high activity. When the active heptapeptide disulfide terminated in a free carboxyl group instead of in an amide, *i.e.,* when a glycine residue replaced the glycinamide residue, as in peptide 3, the activity was also found not to change materially. As the active heptapeptide structure was simplified by omission of first the isoleucyl and then also the glutaminyl residues (peptides 4 and 6), progressive loss in strepogenin activity occurred. Thus the activity of the disulfide of Asp(NH$_2$) - CySH - Pro-Leu-Gly(NH$_2$) was only 20 units per micromole. Furthermore, the lack of not only the isoleucyl and glutaminyl residues but also the asparaginyl residue resulted in a peptide, the disulfide of CySH-Pro-Leu-Gly(NH$_2$), with only a trace of strepogenin activity. Two peptides representing sequences of the active heptapeptide amide but lacking amino acid residues towards the carboxamide end were found to be relatively low in strepogenin activity. These were the disulfides of Ileu-Glu(NH$_2$)-Asp (NH$_2$)-CySH(NH$_2$) and Ileu-Glu(NH$_2$)-Asp(NH$_2$)-CySH-Pro(NH$_2$). It is of interest in this connection that experiments have shown that cleavage of the C-terminal glycinamide residue from arginine-vasopressin by the action of trypsin, which causes loss of pressor activity(14), did not result in the loss of strepogenin activity within the error of the assay. Although several related peptides simpler in structure than oxytocin were found to be considerably active, activity was seen to fall off when certain other structural changes were made. Some specificity of structure is clearly involved in a peptide before it exhibits high strepogenin activity. Thus from the activity of many cysteine-containing peptides and from the slight but real potency of cysteine or

TABLE I. Strepogenin Activity of Peptides Derived from Synthetic Protected* Peptides.

No.	Peptide	Units/μmole†
1.	CyS–Tyr–Ileu–Glu(NH_2)–Asp(NH_2)–CyS–Pro–Leu–Gly(NH_2) (oxytocin)	300
2.	Ileu–Glu(NH_2)–Asp(NH_2)–CyS–Pro–Leu–Gly(NH_2)	250
3.	Ileu–Glu(NH_2)–Asp(NH_2)–CyS–Pro–Leu–Gly	190
4.	Glu(NH_2)–Asp(NH_2)–CyS–Pro–Leu–Gly(NH_2)‡§	35–130
5.	*iso*Glu(NH_2)–Asp(NH_2)–CyS–Pro–Leu–Gly(NH_2)	10
6.	Asp(NH_2)CyS–Pro–Leu–Gly(NH_2)‖	20
7.	CyS–Pro–Leu–Gly(NH_2)	<3
8.	Ileu–Glu(NH_2)–Asp(NH_2)–CyS–Pro(NH_2)	30
9.	Ileu–Glu(NH_2)–Asp(NH_2)–CyS(NH_2)	15
10.	Ileu–Glu(NH_2)–Asp(NH_2)	<3
11.	Glu(NH_2)–Asp(NH_2)	<2

* Peptides 2, 3, 4, and 9 were derived from the corresponding N-tosyl, S-benzyl compounds by reduction with sodium in liquid ammonia; peptides 5, 6, and 8 were derived from the N-carbobenzoxy, S-benzyl derivatives; peptide 10 was obtained from the N-tosyl derivative, and peptide 7 from the S-benzyl derivative. All of the amino acids were of the L-configuration.

† Except for peptides 1 and 2, the peptides were not isolated. The potencies given for the peptides were calculated from the activities obtained by assaying the product resulting from sodium-liquid ammonia reduction of the various protected peptides. The solutions obtained after reduction were treated in 2 ways before assaying. Part was aerated to form the disulfide. Another part was mixed with cysteine in an attempt to maintain the sulfhydryl form of the peptide. (Oxytocin was not assayed in this form.) In each case the activities of the 2 preparations were the same within experimental error.

The potency in units per micromole is expressed for the various sulfur containing peptides, with the exception of oxytocin, on the half-cystine basis.

‡ Assayed without autoclaving. When autoclaved, the compound was almost completely inactive.

§ Dose-response curve steeper than normal.

‖ Dose-response curve sigmoid in shape (see Fig. 1).

cystine, one might argue that for strepogenin activity, only a cysteine or cystine peptide would be required. This argument would be defeated by the inactivity of the disulfide of CySH-Pro-Leu-Gly(NH_2), and by the small activity of the disulfide of IsoGlu(NH_2)-Asp(NH_2)-CySH-Pro-Leu-Gly(NH_2). Similarly one might make a case for a glutaminyl peptide from the activities of oxytocin, arginine-vasopressin(5), the heptapeptide amide disulfide, peptide 3, and peptide 4 [*viz.*, the disulfide of Glu(NH_2)-Asp(NH_2)-CySH-Pro-Leu-Gly(NH_2)]. All these contained a glutamic acid residue and were active. However, the inactivity of performic acid-oxidized oxytocin§(5), S, S′-dibenzyl oxytocin¶(5), glutaminyl asparagine(5), and isoleucyl-glutaminyl-asparagine(5) would negate such an argument. In addition, the slight and aberrant, but nevertheless real, activity of the disulfide of Asp(NH_2)-CySH-Pro-Leu-Gly(NH_2) would be impossible to explain on this basis. Indeed, one can find little justification from these data for saying that the only requirement was a peptide containing any one particular amino acid. What effect rearrangement of a given sequence of amino acids would have on the strepogenin activity of a potent peptide remains to be determined.

Whether these findings may be more generally applicable may have to await the results of studies with organisms other than *L. casei,* since it is known(*cf.* 3,13) that this organism has a less exacting strepogenin requirement than do the group A hemolytic streptococci or mice.

Summary. A number of synthetic peptides representing various sequences occurring in oxytocin have been assayed for strepogenin activity with *Lactobacillus casei.* Several of these simpler peptides have been found to have high strepogenin activity. As the oxytocin molecule was simplified by the omission of the first two amino acid residues at the

terminal amino position, strepogenin activity was not significantly altered. Thus the disulfide of the heptapeptide isoleucylglutaminylasparaginylcysteinylprolylleucylglycinamide had activity comparable with oxytocin. Further shortening of the sequence from the amino end led to the peptide glutaminylasparaginylcysteinylprolylleucylglycinamide disulfide with diminished potency; and when the glutaminyl residue was omitted in addition, activity fell markedly. When amino acid residues were omitted towards the carboxamide end of the active heptapeptide amide disulfide, 2 resulting peptides isoleucylglutaminylasparaginylcysteinamide disulfide and isoleucylglutaminylasparaginylcysteinylprolinamide disulfide had low activities. Cleavage of the C-terminal glycinamide residue in vasopressin by the action of trypsin resulted in little change in strepogenin activity although pressor action was destroyed. The bearing of these findings on the question of nutritional requirements for specific peptides has been discussed.

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Relationship of Physiological Inhibitor of Hyaluronidase to Experimental Poliomyelitis.* (21912)

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An increase in the hyaluronidase inhibitory activity of human and monkey blood after infection with the virus of poliomyelitis has been reported(1). Similar, nonspecific elevations of hyaluronidase inhibitor have been observed to occur in association with many acute systemic changes regardless of whether the diseases were bacterial, viral, or of unknown etiology. The material believed to be mainly responsible for this increased inhibitory activity is referred to as nonspecific or "physiological inhibitor" in contradistinction to the heat stable antibodies produced in response to specific hyaluronidases.

The studies reported herein serve to establish a detailed pattern of "physiological inhibitor" levels in early acute poliomyelitis of monkeys in addition to disclosing a relationship between the route of introduction of the poliomyelitis virus and the subsequent behavior of hyaluronidase inhibitor levels in the sera of these animals.

Materials and methods. Serum inhibitor levels were determined by a modification of the hyaluronidase assay technic of Bachtold and Gebhardt(2). Inhibition is expressed in percentage, the calculations being based upon

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* Aided by a grant from the National Foundation for Infantile Paralysis.

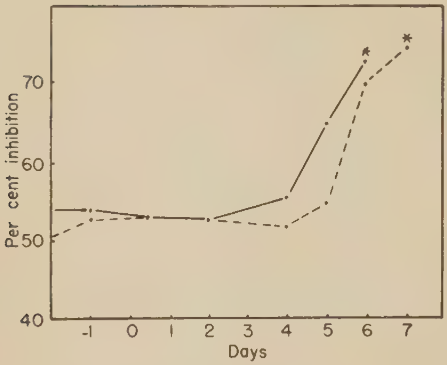

FIG. 1. Typical serum inhibitor patterns following infection with virus of poliomyelitis. —— Intracerebrally infected monkeys. - - - Intranasally infected monkeys. * Appearance of definite paralysis. 0 day = Day upon which virus was inoculated.

differences in reaction rate constants in the presence and absence of monkey sera:

$$\frac{ke - ks}{ke} 100 = \% \text{ inhibition}$$

ke = reaction rate constant of enzyme control
ks = reaction rate constant in the presence of monkey serum

Enzyme and substrate were prepared by the methods of Hahn(3) and Tolksdorf(4) respectively. Serum was obtained from cynomolgus monkeys before and at various intervals after infection with poliomyelitis virus. These samples were quick frozen and stored at −60°C until needed. The entire series taken from any single animal were run simultaneously in order to preserve the relative change in activity between successive samples. In this manner any loss of inhibitory activity due to freezing did not affect the outcome of the test. The Mahoney strain of type I poliomyelitis virus was used throughout the experimental work; the route of inoculation being described under results. Virus neutralizing antibody titers were determined in tissue culture utilizing the method described by Salk(5).

Results. Animals infected by either the intranasal or intracerebral route demonstrated an identical pattern in the rise of serum hyaluronidase inhibitor (Fig. 1). These routes of inoculation are similar because, in either case, the virus enters the brain more or less directly without the appearance of a preparalytic humoral phase. The rise in inhibitory activity from normal to elevated levels is abrupt, with a significant increase in titer (as calculated by a variation of the Student "t" method) just preceding or concomitant with the first symptoms of paralysis (Table I). No increase in virus neutralizing antibody was observed in animals infected by either of these routes before, or up to 2 weeks following paralysis.

Animals infected via the gastro-intestinal tract(6) failed to exhibit a significant rise in

TABLE I. Appearance of Significantly Elevated Serum Hyaluronidase Inhibitor Compared to Course of Experimental Poliomyelitis in Cynomolgus Monkeys.

Monkey No.	Route of infection	Interval from time of virus inoculation until: 1st detectable symptoms, days	Definite paralysis, days	Significant rise in inhibitor, days
1	i.c.	5	5	5
2	"	7	8	6
3	"	5	6	5
4	"	6	6	6
5	"	6	7	5
6	i.n.	5	6	3
7	"	7	7	6
8	"	8	8	5
9	"	6	7	4
10	"	7	9	6
11	g.i.	13	16	None
12	"	9	10	"
13	"	9	10	"
14	"	9	11	"
15	"	8	8	"

i.c. = Intracerebral inoculation. i.n. = intranasal inoculation. g.i. = gastro-intestinal inoculation.

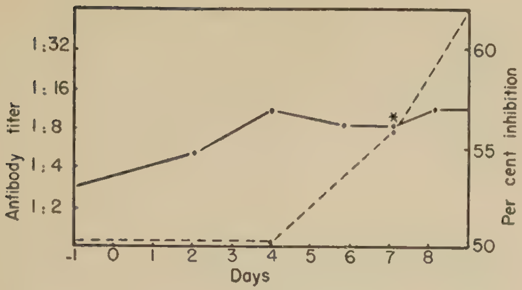

FIG. 2. Hyaluronidase inhibitory and virus neutralizing levels in serum of a monkey selected at random from a group of 20 animals infected gastro-intestinally with poliomyelitis virus. —— Hyaluronidase inhibition. - - - Neutralizing antibody. * Appearance of definite paralysis. 0 day = Day upon which virus was inoculated.

hyaluronidase inhibitor prior to, or at the time of onset of paralysis (Table I). It is noteworthy, however, that in every instance the sera of these animals showed an increasing virus neutralizing capacity over this same period (Fig. 2).

The possibility that virus neutralizing antibody interferes in some manner with the factors responsible for the inhibition of hyaluronidase was considered. It was found that the addition of poliomyelitis immune serum to normal monkey serum in no way lowered the enzyme inhibitory capacity of the latter thus indicating that no direct interaction between antibody and inhibitor occurred.

Comparative inhibitor levels of monkey sera before and after infection with the virus of poliomyelitis are shown in Table II. The maximum levels obtained with the sera of gastro-intestinally infected animals remained well within the limits of analytical error, this value having been calculated to be = 3.1% inhibition.

TABLE II. Comparison of Normal and Maximum Serum Hyaluronidase Levels Observed in Individual Monkeys After Infection with Poliomyelitis.

	% inhibition		Increase in % inhibition	
Monkey* No.	Before inoculation	Following inoculation	actual	relative
1	24	41	17	71
2	59	74	15	24
3	37	60	23	62
4	24	50	26	110
5	32	46	14	44
6	48	69	21	44
7	32	60	28	88
8	32	58	26	81
9	31	52	21	68
10	29	43	14	48
11	22	28	6	27
12	32	35	3	9
13	43	45	2	5
14	38	40	2	5
15	26	26	0	0

* These are the same animals as indicated in Table I.

Discussion. The increased ability of monkey serum to inhibit hyaluronidase in acute poliomyelitis has been studied. Since the factor responsible for inhibition in these animals has been shown to be the heat labile "physiological inhibitor" of hyaluronidase, it can be postulated that this phenomenon is nonspecific for poliomyelitis and is associated in some manner with acute systemic change. For this and other reasons tests of this sort would seem to be of little value as diagnostic tools.

The significance of the observation that gastro-intestinally infected animals, in contrast to those infected via the intracerebal or intranasal routes, demonstrated no early rise in inhibitor is difficult to evaluate. In the search for a common factor with which to relate the increase of inhibition to the route of infection, it was noted that *only* in gastro-intestinally infected monkeys was there an increase in virus neutralizing antibody at or near the time of paralysis. Whether or not this reciprocal relationship between hyaluronidase inhibitor and neutralizing antibody is coincidental remains to be seen. It has been shown not to be the result of a direct interaction between antibody and inhibitor which would have seemed to be a logical possibility.

Summary. The sera of monkeys infected either intracerebrally or intranasally with poliomyelitis virus exhibts an elevated hyaluronidase inhibitory activity at the time of, or shortly before paralysis. Those animals infected by the gastro-intestinal route do not demonstrate a similar increased activity over this same period. A reciprocal relationship between the presence of virus neutralizing antibody and the hyaluronidase inhibitory capacity of the serum is noted.

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Effect of Coramine on Liver and Plasma Protein. (21913)

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N, N - diethylnicotinamide (Coramine) causes a marked increase in the absolute, fat-free liver weight of rats when the drug is fed in a high protein diet(1). Neither nicotinic acid monoethylamide nor some 16 other pyridine derivatives produce this effect(2,3). A similar liver enlargement results if mice are fed Coramine. Such mice have been raised to maturity, have been bred, and have raised young which, in turn, have grown to maturity on the diet(4). The present study was devised to expand upon the above findings and to follow liver and plasma protein changes at various low dietary levels of Coramine.

Materials and methods. Weanling rats, 19 to 24 days old, were placed upon a diet consisting of 25% casein, 55% starch, 15% cottonseed oil and 5% salts(5). Each rat received 1 drop of percomorph oil and a daily dose of 1 ml of brewer's yeast extract (equivalent to 1 g of dried yeast) during the 3- to 6-day feeding period. The rats were an inbred mixture of Sprague-Dawley and Illinois strains. Coramine was mixed into the experimental diet at levels of 0.25, 0.5 and 1% dry weights. After the animals had been on their respective diet for 3 to 6 days, they were bled, the livers were removed and weighed, and a sample (100 to 250 mg) was saved for protein analysis. Nitrogen content of the liver samples was determined with the aid of a Kjeldahl digestion procedure and Conway diffusion cells(6). Total plasma protein in heparinized blood was determined either after digestion of a tungstic acid precipitate of the plasma followed by microdiffusion NH_3 analysis, or directly upon the plasma using a biuret method(7). One one-hundredth ml of plasma from each animal was subjected to paper electrophoresis on an LKB instrument using Munktell No. 20 S filter paper, 0.05 M barbital buffer of pH 8.6. Parallel samples of control and experimental plasmas were run for 5 hours, at room temperature, 11 to 18 milliamperes and 278 to 285 volts. The paper was then dried, stained with bromphenol blue, eluted with 0.01 N NaOH, and color intensity of the eluate was determined in a colorimeter (8).

Results. Weanling rats fed Coramine at the 1% level failed to grow during the short

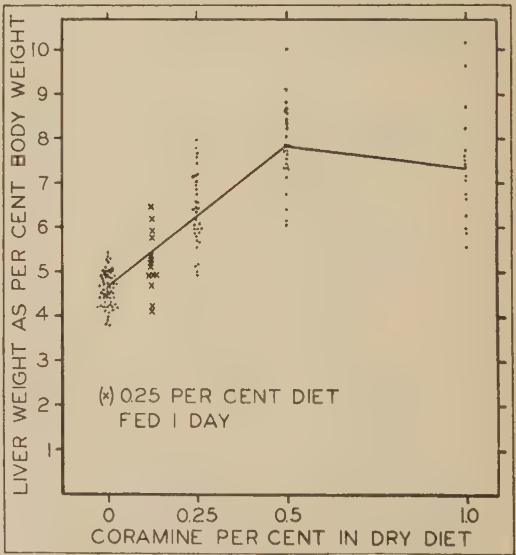

FIG. 1. Effect of feeding various dietary levels of Coramine upon ratio of liver wt to body wt in young rats. Dots and crosses represent individual measurements; line graph is the mean result.

TABLE I. Effect of Dietary Level of Coramine on Liver Growth.

% Coramine in dry diet	No. of animals Coramine	No. of animals Controls	Original body wt, g Coramine	Original body wt, g Controls	Total liver protein (N × 6.25), mg Coramine	Total liver protein (N × 6.25), mg Controls	% increase in liver protein	% probability that increase is due to chance
.25 (fed 1 day)	14	22*	42.6	42.5	637 ± 86†	561 ± 137†	13.5	<10‡
.25	34	22*	41.0	42.5	696 ± 106	561 ± 137	24.0	< 1
.5	24	25	34.7	35.8	594 ± 129	445 ± 84	33.5	< 1
1.0	15	14	38.4	39.1	483 ± 67	433 ± 54	11.5	< 5

* Same control series. † Stand. dev. from the mean. ‡ "T" test(9).

feeding period. Animals on diets containing 0.5% or less of Coramine grew at the average rate of 1 g a day or more. Most of these animals as well as many of the controls failed to consume much diet during the first 2 feeding days, but food consumption, and hence weight gained, was high during the last 2 or 3 days of the feeding period.

The ratio of liver weight to body weight in rats fed Coramine is shown in Fig. 1. Each dot represents an individual measurement. The ratio varies directly with the dietary level of Coramine up to 0.5%. Liver enlargement also occurred in a group of rats which had been accidentally fed the 0.25% Coramine diet for only 1 day even though the absolute consumption of the drug averaged only 8 mg per animal. The rats on the 1% diet did not eat as readily as did rats fed diets containing lower levels of Coramine. This was probably the main reason for the greater variability found for the liver weight to body weight ratio in animals fed the higher concentrations of Coramine.

Table I summarizes the effect of various dietary levels of Coramine on total liver protein. The various groups of experimental animals are compared with their particular control groups. A dietary level of about 0.5% of the drug appears to produce the optimal effect upon liver growth. Although liver weight as % body weight is high for the 1% group, growth was so depressed, due to decreased food intake, that the absolute liver protein is only slightly increased above that of the control series.

Coramine in the diet also causes an increase in plasma protein concentration. Forty-five control plasmas contained an average of 4.96 ± 0.46* g of protein per 100 ml of plasma as compared to the average of 5.19 ± 0.54* g of protein per 100 ml of plasma contained in plasmas from the 87 rats fed the various Coramine diets. Since the packed cell volume of the blood was slightly less in Coramine animals than in controls, the increase in plasma protein probably was not due to dehydration. Although the absolute difference between experimental and control values was not great, the same direction of variation was found in every paired experiment. If checked statistically by means of the "T" test, the difference is significant at less than the 5% level of probability.

Protein patterns of plasma from rats fed 0.5% Coramine or less, were identical with control patterns. The characteristic strong albumin and beta-globulin zones were present with no apparent alterations in mobility. The area beneath the patterns of plasmas from experimental animals was slightly greater than the area beneath control patterns. Occasionally plasma from rats fed 1% Coramine was found which had a decreased albumin component accompanied by an increased beta-globulin fraction.

Summary. N, N-diethylnicotinamide (Coramine), when fed to weanling rats in a 25% protein diet, produced a marked increase in liver size within 3 to 6 days. This enlargement is due, in part, to an average absolute increase of up to 33% in liver protein. The optimal effect is obtained at a dietary level of about 0.5% Coramine. Plasma protein concentrations are slightly higher (5%) in experimental animals than in controls. Paper electrophoretic patterns of the proteins of

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* Standard deviation from the mean.

these plasmas are identical with the patterns of control plasmas. These plasma and liver effects are obtained in a few days when the drug is fed at low dietary levels. Thus it would appear the Coramine acts as a liver growth stimulant in the young rat.

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Nutrition of Brucellae: Utilization of Iron, Magnesium, and Manganese for Growth.* (21914)

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Traces of metallic elements have been incorporated into the various chemically defined media for brucellae, but the quantitative requirements usually have been evaluated only by adding to basal media already relatively contaminated with minerals. A prominent exception is the extensive investigation by Waring *et al.*(1) of the role of metals in the biology of *Brucella suis*. The present study examines the requirement by strain 19 of *Brucella abortus* for Fe^{++}, Mg^{++}, and Mn^{++} in a synthetic medium using 8-hydroxyquinoline as a chelating agent to exhaust the basal medium of these elements.

Methods. Mineral requirements were determined in the chemically defined medium of Gerhardt and Wilson(2), with 0.2% glutamate as the nitrogen source and without the addition of the metals. It was made up in double strength and adjusted with NaOH to pH 7.0 for Fe^{++} and Mn^{++} extraction and subsequently with NH_4OH to pH 9.0 for Mg^{++} extraction. The excess of NH_3 was volatilized during autoclaving. After pH adjustment, the metals were removed by 4 or 5 successive additions of 8-hydroxyquinoline (oxine) and extractions with chloroform until traces of color were no longer visible. Further details are described by Waring and Werkman(3). Conventional spot tests were used to verify the completeness of the extraction process. The desired quantities of metallic ions were then added to the medium as the bivalent sulfates. The pyrex glassware and the glass wool used for plugging were cleaned first with detergent and then with concentrated sulfuric acid saturated with nitrite, rinsed, autoclaved while either filled with or submerged in water, rinsed, and drained. Water used in rinsing and in preparing solutions was doubly glass distilled and oxine extracted.

Suspensions of the inoculum, grown on Albimi Brucella Agar, were washed twice and then standardized turbidimetrically in buffered saline so as to give a final inoculum concentration of 3×10^6 viable cells per ml. A

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TABLE I. Representative Growth Responses of *B. abortus*, Strain 19, to Varying Concentrations of Fe^{++}, Mg^{++}, and Mn^{++}.

Metals added in μg/ml			% light transmittance after indicated hrs of incubation			
Fe^{++}	Mg^{++}	Mn^{++}	24	48	72	96
0	10.0	.10	97	88	78	70
.0050	10.0	.10	97	88	76	70
.010	10.0	.10	95	80	39	27
.10	10.0	.10	97	86	57	44
1.00	10.0	.10	97	87	76	70
.010	0	.10	96	94	89	88
.010	5.0	.10	95	89	40	36
.010	9.0	.10	95	85	34	25
.010	10.0	.10	96	83	30	20
.010	12.0	.10	96	88	38	28
.010	10.0	0	98	86	44	31
.010	10.0	.05	97	84	36	27
.010	10.0	.10	95	81	29	20
.010	10.0	.50	96	86	39	30
.010	10.0	1.0	97	86	45	32

turbidity-count correlation plot was obtained for this purpose, the equation for which is

$$\log G = 2 - 0.194\ N$$

where G = % light transmittance and N = numbers of viable cells per ml in billions. Self-adjusting micropipettes were used to deliver uniform 0.1 ml inoculum volumes. Cultures were grown in 10 ml quantities in optically matched, 20 mm tubes so that growth could be assayed turbidimetrically throughout the growth curve. Verifying plate counts were made at intervals and they compared satisfactorily with the relationship shown in the above equation if the counts were made prior to the maximum stationary phase of growth. Triplicate tubes were incubated in a humidified atmosphere at 35°C on a shaker reciprocating 90 complete strokes per min through 8 cm. The tubes were slanted at 30° and arranged in line with the shaker motion to provide aeration and agitation.

Results. Table I shows the growth response of *Brucella abortus,* strain 19, to various ionic concentrations of iron added to the purified medium, with magnesium and manganese at optimal concentration. In 3 confirmatory experiments, other levels of iron were used, but the data shown here are representative and demonstrate the marked growth response. Levels above 1.0 μg per ml caused visible precipitation and no growth was detectable. The optimal concentration of added iron repeatedly was found to occur at 0.01 μg per ml. Even with the extraction process and no added iron, some growth occurred. Consequently, demonstration of the essentiality of iron was made by serial transfers in the iron-depleted medium. After 3 transfers, growth ceased. It was concluded that iron represents an essential metallic element for growth of the organism.

Table I also includes data representative of those obtained for the growth response to added magnesium ions in the presence of optimal amounts of iron and manganese. As with iron, the effect of added magnesium was quite marked, the optimum occurring at the relatively high level of 10.0 μg per ml. Concentrations above 15 μg per ml caused precipitation and completely stopped growth. Slight growth occurred in the magnesium-deficient medium, but no growth occurred with the second transfer from this culture. Magnesium was concluded to be an essential element for growth of the organism.

The data of Table I show a representative growth response to added manganese ions. Contrary to the preceding results, the response to manganese was comparatively slight and the maximum occurred at 0.10 μg per ml of manganese. Moreover, relatively abundant growth occurred in the manganese-deficient medium and growth was sustained through five serial transfers, although diminishing in level. The results appeared inconclusive as to essentiality of the element, with a stimulatory effect indicated at best.

An experiment was conducted to determine the intereffect of magnesium and manganese, based on their known replaceability in some enzyme reactions. The data are given in Table II. Iron was present in the basal me-

TABLE II. Effect of Intervariation of Concentrations of Mg^{++} and Mn^{++} on Growth* of *B. abortus*, Strain 19.

Mn^{++} added in μg/ml	Mg^{++} added in μg/ml			
	0	1.0	5.0	10.0
0	76	40	31	31
.010	75	40	28	28
.050	78	43	30	23
.10	75	41	27	26
.50	76	42	32	26

* % light transmittance after 96 hr incubation.

dium at the optimum level of 0.01 μg per ml. There appeared to be neither an interdependence nor a sparing effect between the two elements under the conditions of the experiment; that is, growth was greatest at the levels found optimal for each element tested singly.

Discussion. The optimal level and essentiality of iron for growth of strain 19 of *B. abortus* differs from the somewhat higher optimum and apparent stimulatory nature of iron reported(1) for *B. suis* in an oxine-depleted synthetic medium similar to that used here. Such differences, as well as the appearance of growth initially in the depleted media, may reflect the degree of metal depletion, prevention of metal contamination, carry-over of metals in the inoculum, presence of various sequestering compounds in the media, strains used, growth levels reached, or other variables. Even without extraction procedures, stimulatory effects of iron and a comparable optimum for the magnesium requirement have been reported(4,5) for brucellae. The present findings with manganese appear equivocal; if indispensable, the element is required at threshold values below the level of contamination that might still occur in the present procedure.

Summary. The growth response of strain 19 of *Brucella abortus* to added metallic ions was studied in a defined medium depleted of metals by chelation with 8-hydroxyquinoline. Fe^{++} and Mg^{++} were found to be essential, with the optimal responses at 0.01 and 10.0 μg per ml, respectively. The essentiality of Mn^{++} was not demonstrated and the optimum at 0.1 μg per ml was indistinct. No intereffects were shown between varying concentrations of Mg^{++} and Mn^{++}.

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Growth Factors for *Endamoeba histolytica*.* (21915)

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Endamoeba histolytica was first cultivated in the presence of bacteria by Boeck and Drbohlav(1), but has not yet been maintained in pure culture in the absence of bacteria. The problems involved in the cultivation of this protozoan have been reviewed by Anderson *et al.*(2), Porter(3) and Nakamura (4). Jacobs(5) and Shaffer and Frye(6) maintained the amebas in media containing penicillin-inhibited bacteria, although the absence of living bacteria was not claimed. These workers initiated the study of nutritional elements for *E. histolytica* in an environment where bacterial activity was at a minimum. Karlsson, James and Anderson(7) described an assay method for the evaluation of growth factors in single experiments without the necessity of numerous transfers. They found autoclaved bacterial cells to contain a substance which was needed by the amebas for survival. In these studies cultures containing the stimulatory substance had ameba counts of 10 per low power field whereas controls without the growth promoting agent had 1 to 2 amebas per low power field.

The present studies have shown that adenosinetriphosphate (ATP),[†] ribose-5-phosphate,[†] and diphosphopyridine nucleotide (DPN)[†] are growth factors for *E. histolytica.*

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* From a thesis to be submitted as partial fulfillment of requirements for the degree of doctor of philosophy at Boston University Graduate School, Boston.

Materials and methods. The NRS strain of *E. histolytica* growing with a mixed bacterial flora was obtained from Dr. Quentin M. Geiman, Harvard School of Public Health, Boston. Stock cultures of this organism were maintained on a modified Boeck-Drbohlav (1) egg slant medium overlayed with horse serum-Ringers solution. The basal medium in which compounds were tested for their ameba-growth-promoting ability consisted of egg slants in test tubes overlayed with 1.5 ml horse serum-Ringers solution (1:5 sterilized by filtration through a Selas filter), 0.1 ml of 1% glucose, 5 mg rice powder (Difco), and 15,000 units of crystalline penicillin G per ml of medium. To this basal medium was added 0.5 ml of the materials to be tested, either singly or in combination. These tubes were inoculated with *E. histolytica* from a culture grown with a mixed bacterial flora on Ringers-Egg-Serum medium. The bacterial flora was not exposed to antibiotics prior to introduction into the penicillin-containing assay media. The bacterial flora was killed within 4-6 hours when introduced into this medium containing 5,000 units of penicillin G per ml of medium. The inoculum consisted of 2 drops of a 48-hour culture with an ameba count of approximately 7-10 per low power field. Control tubes containing the basal medium were also inoculated. Control tubes containing neither penicillin nor growth factors were also inoculated. These tubes were essentially similar to the stock cultures containing growing associated bacteria. The culture tubes were incubated at 37°C for 48 hours and the ameba counts made by placing small portions of the sediment on a clean slide, covering with a cover glass, and counting the number of amebas per low power field (10 fields from each sample were counted, two samples being taken from each culture tube). Cultures containing penicillin did not contain viable bacteria as determined by inoculating several loopfuls of culture fluid into 150 ml of fluid thioglycollate medium (BBL). At the end of 48 hours these tubes were free of bacterial growth.

Results. Luxurious growth of amebas occurred in media fortified with the growth factors ATP (100 mg/50 ml, sterilized by filtration through a Selas filter), ribose-5-phosphate (100 mg/50 ml, sterilized by autoclaving), and autoclaved bacterial cells (flora present in the ameba stock culture) grown on fluid thioglycollate medium. The results of the population counts are shown in Table I. Subcultivation of the bacteria-free amebas from the basal medium with ribose-5-phosphate, ATP, and autoclaved bacterial cells in the same medium was not possible. However, bacteria-free cultures containing large numbers of amebas could be kept (without subculture) for up to 9 days and successfully subcultured into stock medium containing viable bacteria indicating that the amebas were still alive. Three successful subcultures were possible in media containing the basal medium, ribose-5-phosphate, ATP, autoclaved bacterial cells and DPN (10 mg/10 ml, sterilized by filtration through a Selas filter). In each subsequent transfer the ameba counts progressively became lower until no further growth occurred in the fourth subculture.

Discussion. It appears that we have, in part, supplied the growth factors ordinarily furnished by living bacterial cells. These factors are ribose-5-phosphate, ATP and DPN. The addition of autoclaved bacterial cells may supply growth factors as yet unidentified or they may merely be particulate matter which the amebas engulf. It was thought earlier that an initial reduced environment was essential for the growth of amebas since the addition of small amounts of sodium thioglycollate (BBL) in the presence of autoclaved bacterial cells acted as a stimulus. However, excellent growth of amebas occurred in the media fortified with ribose-5-phosphate and ATP, without the addition of a reducing agent. Apparently once the amebas commence their growth they are able to furnish their own reduced conditions which they probably need for optimal growth.

The fact that the cultures could not be

[†] Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

TABLE I. Effect of Growth Factors on Growth of *E. histolytica* under Bacteria-Free Conditions.

Material assayed	Total No. of determinations	Avg count per low power field at end of 48 hr
Basal (control)	22	1
" + ribose-5-phosphate	6	29
" + adenosine triphosphate	4	69
" + autoclaved bacterial cells	4	40
" + ribose-5-phosphate + ATP	10	94
" + ribose-5-phosphate + ATP + autoclaved bacterial cells	13	190
Growth controls (stock culture)	19	42

carried beyond 3 transfers indicates that additional growth factors must be supplied when there are no bacteria present or that the factors supplied were not at optimal concentrations. It is very unlikely that growth has occurred due to the carry-over of nutrients from the stock culture during the introduction of the inoculum since the inoculum consisted of only two drops and secondly because the basal medium without growth factors did not support growth of the amebas.

Summary. 1. *E. histolytica* has been maintained free of bacteria for 9 days or 3 subcultures in Ringers-Egg-Serum media fortified with the growth factors ribose-5-phosphate, adenosine triphosphate, autoclaved bacterial cells, and diphosphopyridine nucleotide. 2. This method makes available large numbers of amebas (approximately 1 to 1.5 million per ml) free of living bacteria.

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Renal and Hepatic Lipid Alterations in Choline Deficiency: Relationship to Renal Necrosis. (21916)

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It has been known since the work of Best and Huntsman(1) that choline deficiency causes fatty infiltration of the liver. This has been regarded as the result of impaired transportation of fat from liver to depots(2), and necrosis of cells is usually not a prominent feature(3). The next disorder caused by choline deficiency which has received considerable attention is the acute cortical necrosis of the kidneys in young rats(4). In this disorder the renal lesions progress to the stage of generalized enlargement, tenseness, and "hemorrhagic" discoloration. More recently attention has been called to fatty changes in the kidneys of young rats deficient in choline. It has been postulated by Hartroft(5) on the basis of morphological observations that the renal necrosis due to choline deficiency is a result of obstruction of the circulation due to compression of capillaries by swollen, fatty tubular cells. This hypothesis is of interest as it relates to mechanisms of parenchymal cell damage. It also is important because it indicates that choline deficiency may result in accumulation of lipid in an organ other than the liver. It has been suggested recently that choline exerts a "lipotropic" effect not only in the liver and kidneys of rats but also in the cardiovascular system (6). It is not clear from Hartroft's report(5) whether or not the abnormal accumulation of lipid in the kidneys parallels that which occurs in the liver, what the magnitude of the lipid accumulation is prior to the onset of renal necrosis, or how much of the eventual renal enlargement is due to the increase in lipid. If it could be demonstrated that there is a substantial increase in renal lipid and in renal size occurring well before the onset of the characteristic "hemorrhagic" lesions, it would strengthen the evidence that necrosis is a *result* of accumulation of fat and not just another manifestation of cellular damage. Furthermore, it would provide a firmer basis for believing that the fatty changes are a direct effect of choline deficiency, rather than an indirect effect due to damage produced perhaps through some type of circulatory disturbance other than compression of capillaries (7,8).

Methods and materials. Male Sherman and Wistar rats weighing 30 to 40 g at the beginning of the experiments were used. All of the chemical observations were made on Sherman rats. The experimental diet contained 20% casein, 56% sucrose, and 19% Crisco, together with the necessary salts and vitamins (8). Comparable control animals were maintained on the same diet with 0.3% choline chloride added. Groups of animals were *autopsied* at daily intervals for 7 days after starting the experimental diets. The greatest number of observations were made at the end of 4 days just before development of the characteristic renal lesions in the deficient animals, and after 7 days at which time advanced renal lesions were present. The animals were killed by bleeding. After removing renal capsules and peripelvic fat and tissues attached to the other organs, the ratios of the weights of the kidneys and other organs to the weight of the corresponding animal were determined. In some cases the vascular system of the kidneys was studied by infusing India ink into the aorta of anesthetized animals at a point above the emergence of the renal arteries. Paraffin sections from organs fixed in 10% neutral formalin were stained with hematoxylin and eosin or azure and eosin. Frozen sections were stained with Sudan IV or oil red O. In preparing tissues from deficient animals at the end of 4 days for chemical analyses, organs of the occasional animals with definite renal necrosis already present were eliminated since the object was to discover which changes precede the onset of necrosis. The *moisture content* of renal and hepatic tissues was determined as the loss of weight upon heating in an oven at 95-96°C for 18 hours. In the first 2 experiments, pools of tissue were prepared from aliquots of liver and kidney from 8 animals of each group. *Lipid determinations* were made on duplicate pools. The tissue was ground and refluxed gently for 15 minutes with Bloor's mixture consisting of 3 parts ethanol and 1 part ether. After filtration, the tissue residue was extracted with a second portion of solvent, followed by washing of flask and residue. Total lipid was determined gravimetrically as the petroleum ether-soluble portion of the material remaining after evaporation of the original solvent almost to dryness under nitrogen at 60°C. Aliquots of the petroleum ether solution were utilized for *phosphorus determinations* by the method of Fiske and SubbaRow(9), with a modified digestion procedure. A factor of 25 was employed to calculate phospholipid from phosphorus. The lipid residues to be weighed were dried overnight at 60°C and cooled in a desiccator. *Choline determinations* on duplicate pools of tissue were carried out on material extracted by ethanol-ether in 1 experiment, utilizing the method of Glick(10). In the third experiment (Table I), determinations by additional methods were done on the organs of individual rats. Livers and kidneys were homogenized in a Waring blender for 3 minutes with approximately 100 volumes of Sperry's mixture consisting of equal parts of ethanol and acetone. After centrifugation, aliquots of supernatant fluid were withdrawn for the determinations. *Total lipid* was determined by Bragdon's modification of Bloor's colorimetric dichromate oxidation procedure (11). A factor of 1/16 was employed for

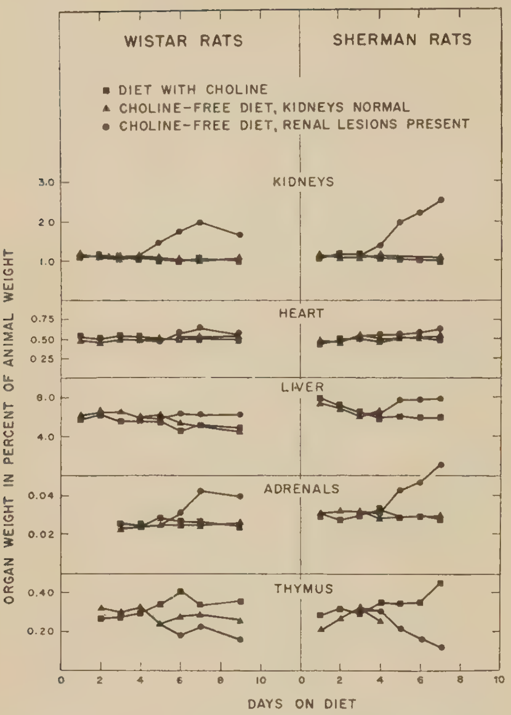

FIG. 1. Change in size of kidneys and other organs in relation to development of characteristic renal lesions of choline deficiency. More than 200 animals are represented in the chart.

converting mg of reduced dichromate to approximate mg of lipid. *Choline* was determined as the periodide(12), after evaporation of the solvent under N_2 and hydrolysis with 20% KOH. *Free and total cholesterol* were determined by a modification of the Schoenheimer-Sperry procedure(13).

Results. a. Size and appearance of kidneys. The kidneys of the great majority of animals on the choline deficient diet could not be distinguished from the control kidneys by gross observations or by examination of microscopic sections until after the 4th day on the diets. At about this time the characteristic renal lesions with necrosis and congestion, manifested in the gross by dark mottling of the surfaces, began to appear. Throughout the pre-lesion period, the tubules of the kidneys from the two groups appeared similar in size of cells and diameter of lumina and the India ink perfusion patterns were similar. Determinations of renal weight relative to body weight failed to demonstrate any consistent difference between kidneys of control and deficient animals. Only with the occurrence of extensive congestion, edema, and necrosis, with the characteristic "hemorrhagic" appearance, was there a considerable increase in relative renal weight (Fig. 1). In animals of the Sherman strain the lesions developed in more than 90% by the 6th or 7th day. Advanced lesions were observed in most groups within 24 hours of the first observation of early lesions, indicating a rapid progression of damage.

b. Lipid, cholesterol and water content. While little stainable fat was observed in frozen sections of kidneys from either deficient or control animals in the early days on the diets, careful observations revealed that at least a few tubules in most of the kidneys from both groups contained collections of small fat droplets. If choline deficiency caused any increase in visible fat during the period prior to the onset of the lesions, the increase was not discernible to us. Fatty changes seemed to develop hand in hand with vascular congestion and tubular necrosis. After development of necrosis in the outer cortex, prominent fatty changes sometimes are observed in less severely damaged tubules in the deeper portions of the kidney. These alterations in the deeper tubules may be secondary to damage in the outer cortex. That fatty changes are not a prerequisite for the occurrence of necrosis is suggested by the occasional observation of extensive necrosis in the presence of very little visible fat.

The determinations of renal lipids in the young rats by chemical methods are summarized in Table I. The lipid analyses indicate that there was no increase in renal lipid —such as might have resulted in widespread compression of renal vessels—up to the approximate time of onset of renal lesions with necrosis (at the end of 4 days). In contrast, there was a considerable increase in *liver* lipids in the deficient animals prior to development of the renal lesions. Plasma cholesterol, phospholipid and neutral fat were decreased. Following the development of the characteristic renal lesions with considerable renal enlargement, there was a definite increase in total lipid per kidney (after 7 days on the de-

TABLE I. Chemical Analyses on Liver and Kidney Tissue.

	Kidney tissue			Liver tissue		
	Choline diet	Choline-free diet		Choline diet	Choline-free diet	
	4 days	4 days	7 days	4 days	4 days	7 days
			Exp. No. 1			
Total lipid (% of wet wt)	3.94 3.86* 4.02	3.87 3.79 3.94	3.39 3.45 3.34	5.33 4.95 5.71	12.56 12.67 12.45	8.02 8.00 8.03
Phospholipid (" " " ")	2.58 2.59 2.56	2.36 2.35 2.37	1.48 1.50 1.45	2.77 2.66 2.88	2.53 2.56 2.51	3.48 3.56 3.39
Moisture (" " " ")	77.6 ±.08†	77.6 ±.21	79.0 ±.22	72.4 ±.36	66.9 ±.10	70.2 ±.22
Size of organ (% of body wt)	1.05±.02	1.09±.02	2.32±.10	6.10±.08	6.23±.21	7.27±.30
			Exp. No. 2			
Total lipid (% of wet wt)	4.52 4.51 4.52	4.38 4.29 4.47	3.66 3.53 3.79	5.07 5.04 5.09	9.96 9.92 10.10	8.28 7.99 8.57
Phospholipid (" " " ")	3.14 3.14 3.13	3.02 3.01 3.03	1.92 1.98 1.85	3.05 3.05 3.05	2.58 2.65 2.50	3.40 3.30 3.50
Choline (mg/g of wet wt)	2.35 2.29 2.40	1.79 1.88 1.71	1.03 .91 1.14	2.69 2.75 2.64	1.38 1.47 1.30	2.53 2.53 2.53
Size of organ (% of body wt)	1.06±.02	1.09±.02	2.28±.25	5.09±.25	5.88±.27	5.71±.26
			Exp. No. 3			
Total lipid (% of wet wt)	3.83±.09	3.68±.11		3.68±.43	9.38±.59	
Choline (mg/g of wet wt)	3.01±.06	2.66±.04		3.12±.09	1.98±.09	
Cholesterol, (mg/g wet wt) total	4.23±.09	4.01±.10		2.40±.14	3.44±.22	
Cholesterol, (mg/g wet wt) free	3.77±.26	3.64±.19		1.86±.08	1.80±.07	
Size of organ (% of body wt)	1.03±.01	1.03±.01		5.60±.18	6.13±.18	

* Results on duplicate pools of tissue from 8 animals, with the mean at the left.
† Mean ± stand. error, of results on 4 to 9 or more individual animals.

ficient diet), though the change was much less than occurred in the liver, and the renal lipid concentration was unchanged or decreased. The increase in total lipid per kidney seemed greater than could be accounted for by congestion and an increased content of blood alone. There was a slight but consistent increase in the previously stable water content of renal tissue and a considerable increase in water per kidney (Table I). It is apparent that the increase in renal size is due largely to increase in substances other than lipid.

c. Choline and phospholipid content. The choline of the kidneys and liver was decreased in the deficient animals before the onset of the renal lesions. Phospholipid was less markedly decreased. By the 7th day, in the presence of advanced renal lesions, the total renal content and the hepatic concentration and content of these constituents had risen considerably from the low levels on the 4th day, suggesting the possibility of an increase in ability of the rats to synthesize choline after an initial period of inadequate synthesis. This might be regarded as a possible explanation of the regression of the acute renal injury while still on the deficient diet. However, this concept is not supported by studies on choline turnover by other investigators(14).

Changes in other organs and renal lesions. Beginning after 1 to 2 days on the choline-deficient diet, the liver was observed to be pale and yellowish. The water content of the hepatic tissue was decreased throughout the period of choline deficiency. The extractable lipid was considerably increased, even after 1 or 2 days on the diet, and reached a peak at about the time of the appearance of the renal lesions (Table I). Necrosis of the liver was not observed.

No difference in relative size of the heart or adrenals occurred between animals of the deficient and control groups prior to development of the renal lesions. It appeared that there may have been some decrease in size of the thymus gland of deficient animals, even before development of the renal lesions. After development of the lesions, definite hypertrophy of the adrenals and decrease in size of the thymus occurred, while heart size remained relatively unchanged. Other late developments included changes in urine, peripheral circulatory failure, changes in muscle tone and intraocular hemorrhages. In addition, hypertension, and arterial lesions, described by other investigators(6,15), were noted in a few animals which developed acute renal injury but survived.

Discussion. During the period prior to the onset of renal congestion and necrosis in the choline-deficient rats (*i.e.*, during the first 4 days), there was no substantial increase in relative renal size, nor was there any over-all increase in lipid content of the kidneys such as occurred in the liver. There was a decrease in renal and hepatic choline prior to the onset of the renal lesions but this alteration was not shown to be the cause of the renal lesions.

Fatty changes, necrosis, edema and congestion of the kidneys all were noted to begin at very nearly the same time. These changes were accompanied in the gross by renal discoloration, enlargement, and an increase in extractable lipid per kidney, but not by an increase in lipid concentration. While it is conceivable that necrosis is actually initiated by, and follows closely in the wake of, fatty infiltration, enlargement of cells and compression of capillaries, such a distinct sequence was not made out in the present study. Clearly the cycle of events leading to cortical necrosis is a rapid one, and one which, in our opinion, cannot be elucidated by the methods available. The observations described here are in agreement in most respects with those of other investigators(16,17).

Renal fatty changes occurring in the first week of choline deficiency apparently have been described only in the rat. Even in the rat, the lipid accumulation is of small magnitude and occurs only in the young animals which develop renal injury, in contrast to the regular early increase in liver lipid to high levels in the majority of species which have been studied. Whether the renal fatty changes in young rats are an effect of a deficiency of choline in the tubular cells or a result of injury through some indirect mechanism, does not seem to have been established conclusively. Fatty changes in the kidneys, in the absence of other evidence of renal injury, have been described in the mouse after 20 days or more on the deficient diet(18). Increased amounts of visible fat also have been observed in the kidneys of adult rats after several weeks of the deficiency (5). While quantitative determinations of renal lipid were not done, and the experiments were complicated by the presence of long-standing fatty livers, these latter observations suggest that renal lipid alterations may occur as a direct result of choline deficiency.

The one pathological change which was consistently observed to precede the development of renal congestion and necrosis due to choline deficiency in rats was fatty infiltration of the liver. While renal lipid did not increase until about the time of the "hemorrhagic" lesions, liver lipid increased progressively throughout the pre-lesion period and reached a concentration of 2 to 4 times normal by the time of onset of the renal lesions. It is quite conceivable that the changes in the liver may have played some part in the production of the renal injury but the present study sheds no light on this problem.

Summary. Before and during the period of development of the characteristic renal lesions of choline deficiency in young rats consisting of a "hemorrhagic" cortical necrosis, observations were made on renal size, morphologic characteristics of the tubules, lipid content and choline content. These observations emphasize the rapid sequence of events in the development of the renal lesions. While the renal lesions are clearly preceded by a considerable increase in hepatic lipid, we were not able to demonstrate the presence of an appreciable degree of renal enlargement or increased renal lipid, in the absence of renal necrosis. After the onset of the renal lesions, with congestion and necrosis, there was an increase in renal size and in total lipid per kidney, but not in renal lipid concentration. Whether compression of capillaries by enlarged, fatty tubular cells is the cause of the renal necrosis, cannot be determined conclu-

sively, in our opinion, by the methods presently or heretofore employed. It is not clear that the renal fatty changes are a *cause* rather than a *manifestation* of injury. Furthermore, it is uncertain whether the fatty changes which accompany the renal lesions result from a metabolic disturbance which might occur in other organs as well, or from some disturbance peculiar to the kidney of the young rat.

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Renal Blood Flow in Anuric Human Subject Determined by Use of Radioactive Krypton 85. (21917)

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The renal blood flow in anuria cannot be measured using the direct Fick methods as these depend on urine formation to determine the amount of test substance excreted. Inert gas diffusion technic using nitrous oxide has been adapted to the measurement of the renal circulation in animal experiments(1). The nitrous oxide results agreed favorably with those obtained using direct Fick method (p-aminohippurate(PAH)) and bubble flow meter in non-anuric dogs(1-2). Inert gas diffusion technic has so far not been used for the study of renal circulation in man.

This report presents a method for the determination of the renal blood flow in man using radioactive krypton 85 (Kr^{85}) as inert gas. Anuric subjects and subjects with normal renal function were studied.

Methods. The Kr^{85} method for the determination of cerebral blood flow(3) has been used with slight modifications. This method is based on the same principle as the nitrous oxide method, *viz.* the exchange of an inert gas between blood and kidney. Access to renal venous blood was obtained by passing a No. 8-9 venous catheter, under fluoroscopic guidance, into one of the renal veins (most often the right) from an antecubital vein. The position of the catheter was assured before, during, and after the examination by comparing the higher renal venous oxygen saturation values with the lower saturation

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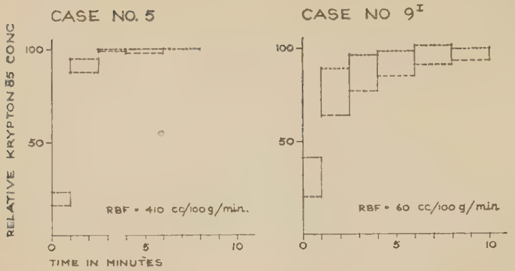

FIG. 1. Relative arterial (- - - - -) and renal venous (– – – –) mean concentrations of krypton 85 during sampling intervals. RBF indicates renal blood flow. Case No. 5 a normal subject, case 9^{I} an anuric subject.

values in the inferior vena cava. Arterial blood was obtained from the femoral or brachial artery through a short polyethylene tube connected to a venous catheter of the same gauge and length as was used in the renal vein. The subjects inhaled atmospheric air containing radioactive Kr^{85} for approximately 10 minutes during which period simultaneous blood samples were drawn automatically from the 2 vessels with a rate of 8 cc/min. The device for blood sampling permitted the collection of integrated arterial and renal venous samples for 1 to 2½ minute periods in immediate succession. The relative concentrations of Kr^{85} in the blood samples were measured(3). These values represent the relative mean concentrations in the collected blood samples during the time intervals chosen. In Fig. 1 the relative Kr^{85} concentrations are plotted against time.

Flow was calculated from the formula

$$RBF_{Kr^{85}} = \frac{100\, R^{t'} S}{\int_0^{t'} (A-R)\,dt - TR^{t'}} \quad \text{cc/100 g kidney/min.}$$

where RBF = renal blood flow in cc/100 g kidney/min.

A = arterial relative concentration of Kr^{85}

R = renal venous relative concentration of Kr^{85}

S = partition coefficient blood:kidney (assumed to be unity)

t' = time of blood:tissue equilibrum

T = time lag between arterial and venous curve (assumed to be 3 sec).

In all subjects the renal extraction ratio of PAH (E_{PAH}) and oxygen was measured

TABLE I. Values for p-Aminohippurate Clearance (C_{PAH}), Renal Extraction Ratio of p-Aminohippurate (E_{PAH}), Renal Arteriovenous Oxygen Difference ($(A-R)O_2$), Total Renal Plasma Flow (RPF_{PAH}), Total Renal Blood Flow (RBF_{PAH}), Blood Flow per 100 g Kidney ($RBF_{Kr^{85}}$), and Oxygen Uptake per 100 g Kidney ($RMRO_{2\,Kr^{85}}$) in 6 Normal Subjects.

								Total kidney perfusion		Perfusion and oxygen uptake per 100 g kidney	
Sex	Age, yr	Wt, kg	Plasma-urea, mg %	C_{PAH}, cc/1.73 m²/min.	E_{PAH}	Hematocrit, %	$(A-R)O_2$, vol %	RPF_{PAH}, cc/min.	RBF_{PAH}, cc/min.	$RBF_{Kr^{85}}$, cc/100 g/min.	$RMRO_{2\,Kr^{85}}$, cc/100 g/min.
♀	43	55	23	—	.90	42	1.6	—	—	350	5.6
♂	41	69	18	550	.89	44	1.5	618	1105	370	5.5
♂	32	65	33	—	.89	44	.5	—	—	760	3.8
♂	45	52	21	534	.89	45	1.35	600	1090	540	7.3
♂	25	68	35	735	.92	44	1.3	800	1430	410	5.3
♂	20	65	32	860	.94	43	1.0	915	1605	770	7.7

(4,5). In 4 of the normal subjects $\text{RBF}_{\text{Kr}}{}^{85}$ was determined in the middle of a 15-minute PAH clearance period. The renal metabolic rate of oxygen (RMRO_2) was expressed by

$$\text{RMRO}_2 = \frac{\text{RBF}_{\text{Kr}^{85}}\,(\text{A–R})\text{O}_2}{100}\quad \text{cc O}_2/100 \text{ g kidney/min.}$$

where $(\text{A}-\text{R})\text{O}_2$ is the renal venous oxygen difference in vol%.

Results. In Table I are presented the results of flow and clearance studies in 6 subjects with normal kidney function. The $\text{RBF}_{\text{Kr}}{}^{85}$ in the normal subjects varied between 350-770 cc/100 g kidney/min. $(\text{A}-\text{R})\text{O}_2$ was 0.5-1.6 vol%, and RMRO_2 was calculated as 3.8-7.7 cc/100 g kidney/min.

In Table II are presented the results obtained in 4 subjects suffering from acute anuria following severe acute barbiturate poisoning with sustained shock. All patients developed severe acute renal failure with a diuresis below 200 cc/24 hours and a 24-hour endogenous creatinine clearance $\leqq$ 1 cc/min. for several days. Plasma urea concentration was considerably elevated. Kidney biopsies were obtained in all patients and showed the changes usually seen in acute ischemic anuria (6). The day of examination is indicated in relation to shock and onset of renal failure. The patients were examined during the anuric phase where the peripheral circulation and blood pressure were normal, and the serum barbiturate concentrations were reduced to low levels. In 2 cases follow-up studies were made.

During anuria $\text{RBF}_{\text{Kr}}{}^{85}$ was 60-360 cc/100 g kidney/min., $(\text{A}-\text{R})\text{O}_2$ 1.0-3.0 vol%, RMRO_2 0.7-3.6 cc O_2/100 g kidney/min., and extraction ratio PAH 0.03-0.19. One subject was reexamined 35 days after onset of disease when C_{Inulin}, C_{PAH}, C_{Urea}, and $C_{\text{Creat.}}$ were normalized, and normal values were found. Another subject was examined twice during the recovery phase: 20 days after the beginning of anuria the renal blood flow and oxygen consumption had increased to about 33 and 50% respectively, of normal values. Normal values were also obtained in this case when clearances had become normal.

TABLE II. Renal Circulatory Data in 4 Anuric Subjects Examined at Various Stages of the Disease. Symbols as in Table I.

Days after onset of		Urine flow, cc/24 hr	Plasma-urea, mg %	Plasma-creat., mg %	24 hr endogenous creatinine clearance, cc/min.	Hemato-crit, %	E_{PAH}	$(\text{A–R})\text{O}_2$, vol %	$\text{RBF}_{\text{Kr}}85$, cc/100 g kidney/min.	$\text{RMRO}_{2\text{Kr}}85$, cc/100 g kidney/min.
Shock	Anuria									
7	5	170	444	11.6	.8	28	.06	2.5	100	2.5
5	3	29	280	11.0	.5	35	.09	1.0	360	3.6
7	5	15	420	18.0	.1	29	.19	1.5	180	2.7
7	4	43	380	10.3	.6	28	.03	1.3	60	.7
38	35	2800	28	.6	92.0	27	.84	1.0	700	7.0
5	4	85	380	11.0	1.0	25	.11	3.0	75	2.2
12	11	1740	638	15.5	4.8	18	–.03	1.7	115	1.9
21	20	3020	46	.90	66.0	20	.75	1.6	190	3.0
52	51	1420	33	.85	105.0	35	.92	1.3	430	5.6

Discussion. The basic assumptions necessary for validating the general Fick equation as applied to the kidney have been discussed in connection with the nitrous oxide method (1). The normal values for $RBF_{Kr}{}^{85}$ in man of approximately 530 cc/100 g kidney/min. may be compared to the normal value in dogs (1) of RBF_{N_2O} of approximately 350 cc/100 g kidney/min. This discrepancy may be due to species difference or to the fact that the dogs were lightly anesthetized. The oxygen uptake per 100 g dog kidney was similar to our results in man. Comparison of the figures for RBF_{PAH} and $RBF_{Kr}{}^{85}$ in Table I indicates a variation in kidney weights in these normal adults of 200 to 350 g (mean weight 270 g). As the Kr^{85} method is essentially a variation of the Fick principle, it is subject to the same limitations. Hence, with high flows the total arteriovenous Kr^{85} difference will be small, and even a small analytical error will alter the result considerably. With flows greater than approximately 400 cc/100 g kidney/min., this error is quite appreciable, and 3 of the experiments reported here had flows exceeding this value considerably.

The renal blood flow in acute renal failure in man has hitherto been studied only using the direct Fick principle by measuring C_{PAH}, E_{PAH}, and hematocrit value(7-8). By the nature of this technic the subjects could not be examined in the anuric phase, and the results obtained in the oliguric phase are for technical reasons open to criticism. The authors(7-8) studied 3 cases of acute oligo-anuria following CCl_4 poisoning, hemorrhagic shock, and incompatible blood transfusion reaction, respectively. They found blood flow values of 3-5% of normal which would correspond to 15-25 cc/100 g kidney/min. as compared to our results of 60-360 cc/100 g kidney/min. There is good agreement between our results and those obtained in experimental anuria in dogs(9).

Summary. 1. Radioactive Krypton 85 was used for the study of renal blood flow and oxygen consumption. 2. Four subjects suffering from acute renal failure were studied during the anuric phase. Renal blood flow and oxygen consumption were reduced to approximately 20 and 25%, respectively, of normal values.

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Isolation and Determination of the Glycoproteins in Cerebrospinal Fluid.* (21918)

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Serum protein bound carbohydrates, the glycoproteins,‡ are greatly increased in such pathological conditions as tuberculosis(1), burns(2), and carcinoma(3,4). No study of these compounds in cerebrospinal fluid has been made except that of Stary *et al.*(5) who determined the protein bound hexoses following precipitation with trichloracetic acid. The paucity of such data is due to the lack of methods. The object of the present study is to describe methods for the determination of the protein bound carbohydrates in spinal fluid.

Experimental. Isolation of protein bound carbohydrates. Since the total quantity of protein in spinal fluid is quite small a relatively large amount of spinal fluid, 100-120 ml, is needed for the separation. The spinal fluid employed was obtained from pneumoencephalograms. From 100 ml of fluid are obtained approximately 30 mg of material containing the alcohol insoluble proteins and the protein bound carbohydrates. The procedure is outlined in Chart 1. If only the protein bound carbohydrate fraction is desired the lyophilized spinal fluid can be extracted with a 1:3 mixture of ether and alcohol the residue dissolved in 1.2% sodium carbonate solution. The rest of the procedure is then carried out as indicated.

The usual methods for the hydrolysis of proteins and polysaccharides employing sulfuric, hydrochloric, or acetic acids, the latter containing hydrochloric acid(7) gave dark colored hydrolysates. The method devised by Glegg and Edinger(8) for the hydrolysis of the polysaccharide of connective tissue, using Permutit Q gave almost colorless hydrolysates. Heavy walled centrifuge tubes that can be tightly closed with steel clamps were used for the separation and hydrolysis.

The best separation of hexose sugars by paper chromatography was obtained when a mixture of butanol, pyridine, benzene, and water, 5:3:1:3, was used in multiple development(9). The presence of D-galactose, D-mannose, and L-fucose was established, the latter sugar present in much lower concentration than the other two. The presence of a hexosamine was established by preparing the dinitrophenyl derivative and chromatographing by the method of Kent *et al.*(10). On the chromatogram the spot lined up with the dinitrophenyl derivative of glucosamine.

Quantitative procedures. (1) Determination of hexoses associated with the glycoprotein. The Badin *et al.*(11) blood glycoprotein method was adapted for use in spinal fluid. Reagents. (a) borosulfuric acid, dissolve 50 g of boric acid in 770 ml of con. sulfuric acid and 230 ml of water, (b) one % aqueous solution of L-tryptophan, (c) stock standard solution, 50 mg each of galactose and mannose dissolved in 100 ml of water. The working standard is prepared by diluting the stock solution so that 1 ml contains 20-50 μg of hexose. The standard contains equal quantities of the 2 hexoses because the chromatographic separation of the hexoses had shown that they were present in approximately equal amounts.

Procedure. To 10 ml of cold, absolute ethanol, in a 15 ml centrifuge tube, add, dropwise, 2 ml of spinal fluid, cover with parafilm and refrigerate overnight. After centrifugation the precipitate is washed twice with 80%

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† Portions of this work were included in a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science.

‡ The term "glycoproteins" is used to indicate all the proteins containing carbohydrates found in the spinal fluid, it includes the glycoproteins and seromucoids according to the classification of Winzler (6).

CHART 1. Isolation of Protein Bound Carbohydrates from Spinal Fluid.

100 ml fluid, lyophilized, extracted with 3:1 acetone, ether	
↓	↓
Residue extracted with 95% ethanol	(Extracts contain lipides)
↓	↘
Residue extracted with 70% ethanol	(Extract contains some lipides, all free sugars)
↓	↘
Residue dissolved in 0.2 ml 1.2% Na_2CO_3 then precipitated with 10 ml ethanol, centrifuged, supernatant discarded	(Extract contains salts, some organic compounds as yet unidentified)
↓	
Centrifugate, containing glycoprotein, hydrolyzed with 2 g Permutit Q and 5 ml water	
↓	↘
Resin eluted with 0.5 N HCl and the eluate evaporated to dryness, dissolved in .25 ml ethanol; used for chromatography of hexosamines	Supernatant lyophilized, dissolved in .25 ml methanol, and used for chromatography of hexose sugars

ethanol; the repeated washing is essential to insure complete removal of the free sugars. After dissolving the precipitate in 1.0 ml of water the tube is placed in an ice bath, 4 ml of the borosulfuric acid and 0.5 ml of the tryptophan solution are added. The tube, covered with a marble, is then heated 15 minutes in a boiling water bath. After chilling for 2 minutes and standing at room temperature for 15 minutes the colored solution is read in a Klett-Summerson photoelectric colorimeter using filter 540. The color follows Beer's law within the limits investigated, 5-50 μg. A water blank is put through the same procedure. Precision of the method was established by determining the hexoses in the glycoproteins in 6 replicates of the same fluid, the average and standard deviation was 16.1 ± 0.4 mg per 1000 ml. (2) Determination of hexosamine associated with the glycoproteins. The determination is made upon the alcohol precipitated glycoproteins by an adaptation of the Elson-Morgan method(12,13). Reagents. (a) acetyl acetone, 0.2 ml dissolved in 10 ml of 0.5 N sodium carbonate, (this solution must be prepared just prior to use); (b) Ehrlich's reagent, 0.8 g p-dimethylaminobenzaldehyde dissolved in 30 ml each of absolute ethanol and con. hydrochloric acid, (c) standard glucosamine solution containing 20 μg of the hydrochloride per ml water. Glucosamine is used because the isolation procedure had shown it to be the only hexosamine present in the glycoproteins. It may be noted, however, that equimolar solutions of glucosamine and galactosamine yield the same optical densities in the colorimetric procedure.

Procedure. The glycoproteins are precipitated with ethanol as described in (1) using a heavy wall centrifuge tube that can be sealed with a clamped stopper. To the precipitate is added 1.0 ml of N hydrochloric acid, the tube is closed and heated in a boiling water bath for 4 hours. After cooling, the solution is neutralized with 1.0 N sodium hydroxide and diluted to 4 ml with water, 2 ml of the acetyl acetone solution are added and the tube placed in a boiling water bath for 20 minutes. After cooling the tube to room temperature 3 ml of ethanol and 2 ml of Ehrlich's reagent are added and it is allowed to stand for 45 minutes. The colored solution is read in a Klett-Summerson colorimeter using filter 540. The factor for the conversion of glucosamine hydrochloride to glucosamine is 0.829. The precision of the method was determined by the analysis of 4 replicate samples, the maximum deviation from the mean was 2.4%. (3) Determination of neuraminic or sialic acid. The methods of Böhm *et al.*(14) and Blix (15) for the determination of this compound were adapted for use in spinal fluid.

Reagents. (a) Dissolve 0.5 g of orcinol in 200 ml of con. hydrochloric acid and 50 ml of water, (b) 0.07 M ferric chloride dissolved in 200 ml of con. hydrochloric acid and 50 ml

TABLE I. Protein Bound Carbohydrates in Spinal Fluid.

	Non-demyelinating diseases		Multiple sclerosis			Brain tumors	
	No. of cases	Avg ± stand. dev.	No. of cases	Avg ± stand. dev.	P	No. of cases	Avg ± stand. dev.
Gamma globulin hexose, mg/L	10	1.8 ± 0.8	20	7.1 ± 3.0	.05		
Gamma globulin protein, mg/L	10	38.0 ± 15.0	20	127 ± 38	.05		
Ratio: $\frac{\text{Hexose}}{\text{Protein}} \times 100$		5.5 ± 2.6		5.6 ± 1.3			
Gamma globulin hexose, mg/L	8	2.4 ± 1.4	6	7.5 ± 3.6	.1	3	12.3 ± 7.0
Glycoprotein hexose, mg/L	8	11.8 ± 2.8	6	27.2 ± 16.0	.02	3	106.6 ± 88.0
Ratio: $\frac{\text{G.G. hexose}}{\text{G.P. hexose}} \times 100$		20.0 ± 3.0		31.0 ± 11.9	.01		13.4 ± 3.1
Glycoprotein hexosamine, mg/L	14	4.2 ± 1.8					
Glycoprotein neuraminic acid, mg/L	10	2.4 ± 0.9					

of water. *Procedure.* To 10 ml of spinal fluid add 2.5 ml of 70% trichloracetic acid solution. Centrifuge, and to the precipitate dissolved in 5 ml of 0.9% sodium chloride solution add 3.5 ml of the orcinol solution and 0.5 ml of the ferric chloride solution. The stoppered tubes are heated in an oil bath at 108-110°C for 10 minutes. After cooling to room temperature the color is extracted with 5 ml of isoamyl alcohol. The colored alcohol extract is read in a Coleman spectrophotometer at wave length 570. The values were calculated from the curve given by Böhm (14). The precision of the method, based on the analysis of 4 replicates showed that the maximum deviation from the mean was 4%. (4) Determination of gamma globulin and associated carbohydrate. Gamma globulin is precipitated and determined by the method of Roboz *et al.*(16). For the estimation of the associated carbohydrate the precipitated globulin is dissolved in 1 ml of 1.25% sodium carbonate solution and hexose and hexosamine determinations can be performed according to (1) and (2) above.

Results. The values are expressed as mg per 1000 ml of spinal fluid, and the standard deviations are included with the average values. The hexose content of the gamma globulin fraction and the total gamma globulin content of the spinal fluid and the ratio of the two in multiple sclerosis and also in non-demyelinating diseases are given in Table I. The probability, P, as a test of the significance of the difference between demyelinating and non-demyelinating conditions is included. Similar comparisons between the hexose content of the glycoproteins and of the gamma globulins are also given in Table I. Determination of the hexose content of the glycoprotein was made on 8 cases of brain tumor, the average value was 71 ± 57 mg per 1000 ml, however both determinations were made on only 3 cases and the results are included in the table merely to indicate the range of values.

While the gamma globulin content of the spinal fluid is significantly higher in multiple sclerosis than in non-demyelinating diseases and the gamma globulin hexose is likewise elevated, the ratio of the two is the same in both cases. However the ratio is affected when the comparison is made between the gamma globulin hexose and the glycoprotein hexose. Recently Weimer *et al.*(17) reported that, after immunization, a significant increase occurred in the polysaccharide moiety of the gamma globulin in blood serum. The increase in polysaccharide merely reflects the increase in gamma globulin and not an enrichment of its carbohydrate content.

No attempt has been made, as yet, to determine differences in the hexosamine content of the glycoproteins in various pathological conditions. The values on 14 cases are given in Table I. In 5 cases sufficient spinal fluid was available for the determination of both the hexose and hexosamine content of the glycoproteins. It was found, on the average, that the ratio of hexose to hexosamine was 71 to 29. In blood serum Winzler(6) found that the ratio was 59 to 41.

It is probable that the neuraminic acid first isolated by Klenk(18) from gangliosides is either the same compound or is closely related to the sialic acid isolated by Blix(19) from submaxillary mucin. Both compounds have the same rotation, give identical color reactions and liberate carbon dioxide when heated with mineral acid. The colorimetric methods used for their determination in blood serum were satisfactorily adapted for use in spinal fluid and the values obtained are included in Table I. No attempt has been made, as yet, to study various pathological conditions.

Summary. 1. A procedure is described for the isolation of the glycoproteins from spinal fluid that also separates the lipides and free sugars. The carbohydrates in the glycoproteins were identified by paper chromatography, mannose, galactose, fucose, and glucosamine were found. 2. Methods are given for the quantitative estimation of the hexose and hexosamine associated with the glycoproteins and the gamma globulin, and also the neuraminic acid content of the spinal fluid. 3. An increase in gamma globulin hexose was found in multiple sclerosis. The ratio of gamma globulin hexose to glycoprotein was likewise increased. However the ratio of hexose to gamma globulin was not different from that found in non-demyelinating diseases, indicating that the hexose increase merely reflected the increase in gamma globulin.

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Effect of Hydrocortisone on Acute Pentobarbital Toxicity in Mice.* (21919)

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The problem of managing patients with acute barbiturate poisoning has grown steadily during the last decade. The reported mortality rate in large series varies between 8% and 14%(1,2). The main treatment administered in the majority of cases consists of one or more of the analeptic drugs such as picrotoxin, amphetamine, nikethamide, etc(2) along with general supportive therapy. Although there appears to be fair agreement on the use of such analeptics, a few reports(3-5) suggest that analeptics are at times harmful in this condition. Thus it has been postulated that in acute barbiturate intoxication there is marked anoxia of the brain with intracellular edema and analeptics might make the patient worse by increasing the oxygen demand beyond the available supply(4). Although it is known that the adrenal cortical hormone increases significantly the survival rate of normal rats exposed to standardised acute altitude tolerance test(6), it may not be useful in the treatment of barbiturate poisoning as these steroids themselves produce anesthetic effect when administered in large doses(7,8). However, mice given barbitone, ascorbic acid, and pituitary adrenocorticotrophic hormone were said to have a smaller mortality than controls given only barbitone; but the action of the hormone alone was not mentioned(9). Recently reports have been published concerning the use of adrenal hormones in barbiturate poisoning(10). The present study was undertaken to find out the effect of hydrocortisone on acute pentobarbital toxicity.

Method and material. A total of 100 mice weighing between 20-30 g were divided into 8 groups of ten mice each, except in Experiments I and III which included 20 animals in each group.

Both pentobarbital and picrotoxin were given intraperitoneally. Experiment I was done to find the LD50 for pentobarbital. In Experiment II picrotoxin alone, in doses of 0.05 mg/10 g body weight, was tested. In Experiments III, IV and V the LD50 for pentobarbital was determined again, but in animals given picrotoxin a few minutes after the pentobarbital when the animal became inactive.

TABLE I. Effect of Picrotoxin and Hydrocortisone on Pentobarbital Toxicity in Mice.

Exp. No.	Dose, mg/10 g body wt	Total No. of mice	Mortality, %
I	Pentobarbital, 1.5 mg	20	50
II	Picrotoxin, .05 mg	10	0
III	Pentobarbital, 1.75 mg + picrotoxin, .05 mg	20	40
IV	Pentobarbital, 2.0 mg +picrotoxin, .05 mg	10	50
V	Pentobarbital, 2.5 mg + picrotoxin, .05 mg	10	100
VI	Hydrocortisone, 1 mg injected	10	0
VII	Hydrocortisone, 1 mg inj. 2 hr prior to + pentobarbital, 1.75 mg	10	100
VIII	Hydrocortisone, 1 mg given orally 2 hr prior to + pentobarbital, 2.0 mg + picrotoxin, .05 mg	10	80

In Experiment VI hydrocortisone alone, in doses of 1 mg/10 g body weight, was given intraperitoneally. In Exp. VII it was administered 2 hours prior to the pentobarbital injection. Group VIII received a similar dose of hydrocortisone orally 2 hours prior to the pentobarbital injection which was then followed by picrotoxin.

Results. Table I shows the results of the 8 experiments. Comparing Experiments III and IV it is obvious that the LD50 of pentobarbital increased from 1.5 to 2.0 mg/10 g body weight on treating the animals with picrotoxin. That picrotoxin given in such doses was harmless is shown by Experiment II. This amount of picrotoxin did not prevent a high mortality in mice that received 2.5 mg/

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10 g body weight of pentobarbital (Experiment V).

From Experiment VI it appears that hydrocortisone given alone in doses of 1 mg/10 g body weight had no toxic effect. But it failed to protect the mice from the acute toxicity of pentobarbital injected in doses of 1.75 mg/10 g body weight, the mortality in this experiment being 100%. It is interesting to note that when hydrocortisone was used along with picrotoxin and 2.0 mg/10 g body weight of pentobarbital the mortality was 80% (Experiment VIII).

Discussion and conclusions. On analysing our results it is obvious that hydrocortisone given alone cannot act as a substitute for the analeptic therapy of acute barbiturate poisoning. All the mice in Experiment VII that received hydrocortisone and 1.75 mg/10 g pentobarbital died, while in a similar experiment where the mice were given picrotoxin instead of hydrocortisone the survival rate was 60% (Experiment III). These results are statistically significant, the value of Chi-square being 7.6. This is not surprising since it is well accepted that picrotoxin can act as a good antidote for barbiturate toxicity in animals (11).

In Experiments IV and VIII, the mortality in the group that received pentobarbital and picrotoxin was 50%, while in Experiment VIII where the mice were also given hydrocortisone prior to the pentobarbital and picrotoxin the mortality was 80%. From these results it seems that hydrocortisone has no value and may be harmful. However the results are not statistically significant, the value of Chi-square being less than 1.

Findings in experiments with mice cannot necessarily be applied to conditions which prevail in human cases.

Summary. The LD50 for pentobarbital given intraperitoneally in mice was determined. Administration of picrotoxin within a few minutes of the pentobarbital injection decreased mortality so that the LD50 for the pentobarbital was higher in animals receiving picrotoxin. On the other hand, orally or parenterally administered hydrocortisone did not decrease mortality and seemed to increase susceptibility to pentobarbital.

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Lysis of Collagen by Human Gingival Bacteria.* (21920)

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Inflammatory lesions in the tissues that surround and anchor the teeth, *i.e.*, in the periodontium, are very common in man. Histologic examination of specimens obtained from such cases of periodontitis reveals destruction of the collagenous fibers that fix the teeth to their surrounding tissues. It has been suggested that enzymes derived from polymorphonuclear leukocytes are responsible for this breakdown of collagen(1). However, many of the bacteria that reside on the gingivae are proteolytic(2-4). It was relevant, therefore, to investigate the capacity of such bacteria to attack collagen and thereby contribute to the destruction of the gingival and periodontal collagenous fibers. Although much work has been done on so-called collagenases(5-7), only recently has a distinction been clearly established between true collagenases, that digest native collagen but not other proteins, and other proteases, that commonly have no effect on intact collagen(8,9). The present investigation was concerned only with determining whether or not human gingival bacteria could digest collagen that was as nearly as possible in its native state.

Materials and methods. Substrate. In order to simulate the situation *in vivo* as closely as possible, collagen in the form of Achilles tendon from freshly slaughtered cattle was employed as substrate. Pieces averaging 135 mg wet weight were placed in sterile test tubes and stored in the frozen state. Prior to their use for the determination of collagenolytic activity, they were sterilized by exposure for 18 hours at room temperature to an atmosphere containing ethylene oxide. Thereafter, 10 ml of broth was added aseptically to each tube. In each test, sterility was confirmed by the failure of microbial growth to appear in an uninoculated tube. *Broth.* Since the liberation of hydroxyproline from tendon was to serve as the measure of collagenolytic activity, it was imperative to employ a broth containing as little hydroxyproline or interfering substances as possible. At the same time, it was necessary that the broth should support the growth of as many kinds of gingival bacteria as possible and that it should favor the development of proteolytic activity. A broth of the following composition was found to be suitable: Trypticase (Baltimore Biological Laboratory), 17.0 g; yeast extract (Difco), 2.5 g; casein (Schering-Kahlbaum), 1.0 g; sodium chloride, 5.0 g; dipotassium phosphate, 2.5 g; magnesium sulfate (septahydrate), 0.05 g; distilled water, 1000 ml. The pH was adjusted if necessary to 7.1. The broth was sterilized in an autoclave at 121°C for 20 minutes. *Gingival samples.* Subgingival accumulations were collected from 40 adults, selected only for the presence or absence of chronic marginal gingivitis, in a manner described previously(10). Each subject was sampled only once. The material from each individual was distributed into tubes of broth, as follows. *Tests for collagenolytic activity.* For each test, 5 tubes of broth were employed. Tubes 1, 2 and 3 contained tendon also, as described above. Tubes 4 and 5 contained broth only. Tubes 1, 2 and 4 were inoculated with the subgingival material. Tubes 3 and 5 were not inoculated but served as control on the sterility and stability of the tendon and as blank, respectively. Tube 1 was incubated aerobically for 7 days at 37°C. The remaining tubes were incubated anaerobically in an atmosphere of 95% nitrogen and 5% carbon dioxide for 7 days at 37°C. Preliminary tests showed that if lysis of collagen was not demonstrable by the seventh day, it was unlikely to become demonstrable after longer incubation. At present, the most specific indicator of collagenolysis is the liberation into

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solution of peptides containing hydroxyproline. Accordingly, at the end of the incubation period, all tubes were centrifuged and the supernates were analyzed for hydroxyproline. The latter was not demonstrable, however, until the supernates were subjected to acid hydrolysis. To 2 ml of each supernate were added 2 ml of 12N HCl. Hydrolysis was carried out in sealed tubes at 121°C for 3 hours. The hydrolyzates were neutralized with NaOH. Hydroxyproline was determined on aliquots by the colorimetric method of Martin and Axelrod(11), using a Klett-Summerson photoelectric colorimeter with the green filter. Under the conditions of the present experiments, the minimal significant increase of hydroxyproline was judged to be 3 μg per ml of supernate.

Results. The nominal hydroxyproline contents of tube 3 (tendon without organisms) and tube 5 (broth only) differed at most by 1 μg per ml. This result showed that the collagen was completely stable when incubated alone in the broth for 7 days. The hydroxyproline content of tube 4 (organisms without tendon) was always slightly lower. This result showed that the mixed cultures contributed nothing to the final result but rather consumed part of the color-producing material of the broth.

The collagen was attacked in the cultures from 13 of 19 persons exhibiting chronic marginal gingivitis but in only 3 of those from 21 persons whose gingivae were clinically normal. This difference would be expected to occur by the chance of fortunate sampling only once in 1500 times. Only the aerobic cultures were collagenolytic in 6 tests and only the anaerobic in 2 tests, whereas both cultures were collagenolytic in 8 tests. In Table I, the data are arranged according to the amount of hydroxyproline liberated. The results indicate that on the average considerably more hydroxyproline was liberated by the mixed cultures derived from the individuals with gingivitis than from those having clinically normal gingivae. Furthermore, even though slightly more aerobic than anaerobic cultures were collagenolytic, between 100 and 170 μg of hydroxyproline per ml were liberated by 4 of the anaerobic cultures, whereas only one aerobic culture approached this order of activity.

TABLE I. Liberation of Hydroxyproline from Tendon by Mixed Cultures of Gingival Bacteria.

Increase of hydroxyproline in supernate, μg/ml	No. of cultures			
	Gingivitis group		Normal group	
	Anaerobic	Aerobic	Anaerobic	Aerobic
101-170	4	1	0	0
51-100	0	3	1	0
6- 50	2	5	1	1
3- 5	2	2	0	2
0- 2	11	8	19	18

In order to make possible conversion of the data into amounts of tissue or collagen destroyed, 6 pieces of tendon were dried to constant weight (average = 40 mg) and each was placed in 2 ml of broth. Hydrolysis and determination of hydroxyproline was carried out in the usual manner. The average content of hydroxyproline was 10%. Since collagen from bovine Achilles tendon contains on the average 13% of hydroxyproline(12), it was estimated that 80% of the solid matter of the tendon consisted of collagen. In terms of destruction of tissue, therefore, an increase of 125 μg of hydroxyproline per ml of culture supernate indicates the destruction of 12.5 mg of dry tendon or of 10 mg of collagen, since there were 10 ml per tube. These amounts correspond to a breakdown of 30% of the average piece of tendon. The data summarized in Table I show that in the majority of instances, from 10 to 30% of the collagen was digested, liberating much more than the minimal significant amount of hydroxyproline, 3 μg per ml, which corresponds to 1% of the average sample of tendon. More precise quantitation was considered to be unnecessary for these experiments. Fig. 1 shows the complete disruption of normal structure that accompanied such partial collagenolysis of a piece of tendon by one of the mixed cultures of gingival bacteria. In 2 cases, incubation was continued until no discernible tendon remained, *i.e.*, for 65 and 78 days, respectively. The analyses for hydroxyproline indicated that no significant amount of this amino acid was destroyed by the prolonged bacterial action.

As a control test of the substrate, 6 pieces of tendon were incubated in 10 ml portions of

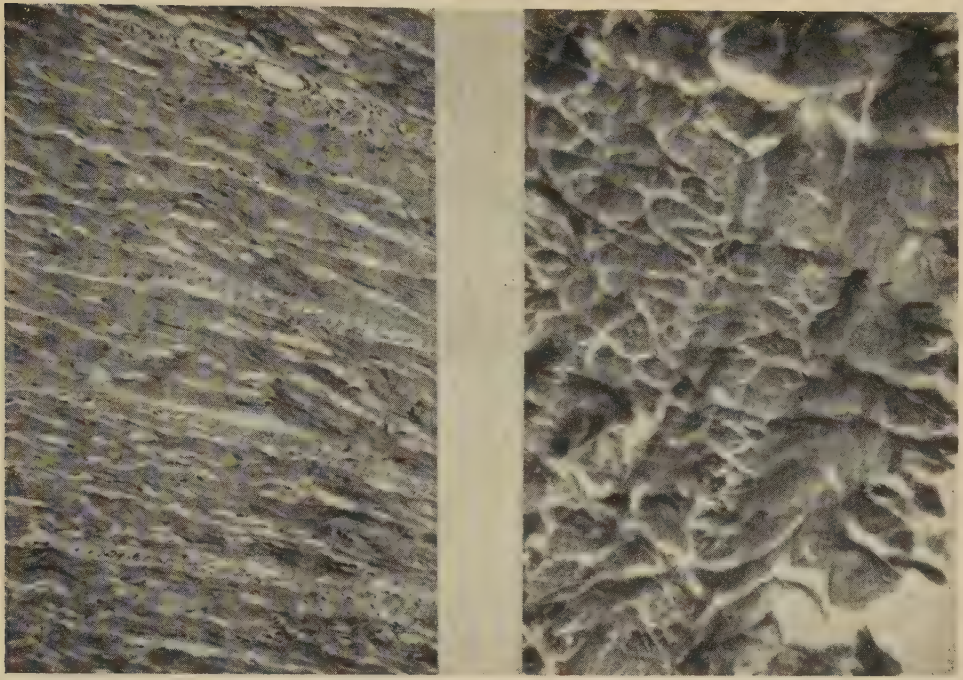

FIG. 1. Left, normal tendon; right, tendon attacked by gingival bacteria. Both specimens were fixed in Zenker's fluid, embedded in paraffin, cut at 8μ, stained with hematoxylin and eosin, and photographed at 98 × magnification.

broth containing from 10 to 60 mg of a crystalline preparation of trypsin. Even after 7 days at 37°C, no liberation of hydroxyproline could be detected. The potency of the trypsin was demonstrated by incubating 5 portions of 50 mg of azocoll(6) suspended in 10 ml portions of distilled water with from 10 to 50 mg of the trypsin. After only 30 minutes at 37°C, release of dye from the azocoll was evident in all tubes.

A number of proteolytic bacteria isolated from the collagenolytic mixed cultures have been tested in the manner described but none has been found to be active on the collagen in tendon.

Discussion. The fact that mixed cultures of human gingival bacteria are capable of attacking presumably native collagen establishes the possibility of the existence of collagenases, as a distinct class of proteases, in accumulations about the teeth. The use of as nearly intact collagen as possible in these tests supports the idea that the collagenous fibers of the periodontium can be broken down by the action of gingival bacteria. The greatly increased incidence of collagenolytic cultures from subjects having chronic marginal gingivitis has a similar significance. The relatively slow action of the bacteria upon tendon could mean that the destruction of the collagen was nonspecific. However, 2 days or longer were required for the complete digestion of similar pieces of tendon by a purified and concentrated preparation of collagenase from *Clostridium histolyticum*. The resistance of the substrate to trypsin also increases the likelihood that a specific collagenase was present in the mixed cultures of gingival bacteria. The collagen could not have been hydrolyzed or rendered susceptible to the action of proteases by exposure to acid conditions (13), for the pH of the cultures was always between 7.1 and 7.6. The failure to date of individual strains of proteolytic bacteria isolated from such mixed cultures to lyse collagen might signify that the process requires the synergistic action of enzymes from several kinds of microorganisms.

A purified preparation of collagen might have been used with some advantage, since the collagen in tendon may be less accessible owing to combination with mucopolysaccharides or unidentified substances. However, the various processes available for the purification of collagen may well increase its susceptibility to enzymatic hydrolysis(13,14). Furthermore, the use of tendon should make the results more significant for the situation *in vivo*. Even so, the methods used in the present investigation are only an approximation of conditions *in vivo*.

Summary. Collagenolytic activity was demonstrated in mixed cultures of gingival bacteria from 13 of 19 individuals with chronic marginal gingivitis but from only 3 of 21 individuals with clinically normal gingivae. This difference is statistically valid. The use of native collagen as a substrate, in the form of tendon, and the determination of the liberation of peptides containing hydroxyproline as an indication of collagenolysis are believed to make the results significant for the situation *in vivo*. Consequently, it is inferred that gingival bacteria may contribute to the destruction of the collagenous fibers of the periodontium seen in periodontitis.

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